FINAL REPORT

Contract No. DAMD17-89-C-9050
A Medical Research and Evaluation Facility (MREF) and Studies
Supporting the Medical Chemical Defense Program

on

TASK 92-28:
CHARACTERIZATION OF THE ANTICYANIDE EFFECT OF METHEMOGLOBINEMIA INDUCED BY CANDIDATE PRETREATMENT DRUGS IN AN ANESTHETIZED ANIMAL MODEL

to

U.S. ARMY MEDICAL RESEARCH
AND MATERIEL COMMAND

September, 1996

by

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GOOD LABORATORY PRACTICES COMPLIANCE STATEMENT

To the best of my knowledge, all aspects of this study were conducted in compliance with the U.S. Food and Drug Administration's Good Laboratory Practices regulations (21 CFR Part 58).

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10-23-96
Date

QUALITY ASSURANCE STATEMENT

This study was inspected by the Quality Assurance Unit and reports were submitted to the study director and management as follows:

Phase Inspected	Date Inspected	Date Reported to Study Director	Date of Report to Management
Protocol Review	7-30-93	7-30-93	8-20-93
Formulation Preparation	9-30-93	10-1-93	10-1-93
Anesthetization of Test System	9-30-93	10-1-93	10-1-93
Blood Collection	9-30-93	10-1-93	10-1-93
Test Article Administration (IV)	9-30-93	10-1-93	10-1-93
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Test Article Analysis	9-30-93	10-1-93	10-1-93
Compound Weights	11-30-93	11-30-93	11-30-93
Formulation Preparation	11-30-93	11-30-93	11-30-93
Standard Preparation	11-30-93	11-30-93	11-30-93
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Study File Audit	12-30-93	12-30-93	1-4-94
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Blood Collection	8-10-94	8-10-94	9-1-94
Blood Analysis	8-10-94	8-10-94	9-1-94
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Final Report Audit	7-22-96	7-22-96	9-20-96

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Health Division

EXECUTIVE SUMMARY

Task 92-28 was conducted for the U.S. Army Medical Research and Materiel Command (USAMRMC) to determine the protective efficacy provided by low methemoglobin (MHb) levels (less than or equal to 10 percent) against a continuous sodium cyanide (NaCN) infusion, and to compare and quantitate the efficacy of long-acting and short-acting MHb-forming compounds in anesthetized canines. In addition, three MHb-forming compounds were evaluated in pharmacodynamic experiments to determine the time course of MHb levels. Two MHb-forming compounds were compared in multiple-dose studies. The primary endpoints for assessing the toxic effects of NaCN were time to respiratory arrest (TRA) and amount of NaCN infused. Time to respiratory arrest was defined as the elapsed time between the beginning of NaCN infusion and the stopping of the infusion when no functional breaths had been observed for 10 sec. Another endpoint was MHb levels in heparinized whole blood. Secondary endpoints varied depending upon the phase of the study, but included the following: time to peak heart rate and peak shift from baseline; time to peak respiratory rate and peak shift from baseline; average total cyanide ion (CN^-) level after respiratory arrest (RA); and peak free plasma CN^- and time to peak. The task was conducted in phases, using animals in multiple phases to reduce the number of animals required, and using results of previous phases to assist in selection of pretreatment (compounds given prior to challenge with NaCN) doses and times for initiation of NaCN infusion.

Phase I experiments were conducted to determine the feasibility of repeated NaCN infusions in the same animal with a minimum one-week washout period. Secondary objectives were to determine the variability in TRA and the replicability of the procedure, and to assess the development of trends in the data over repeated experiments. Four naive anesthetized animals were connected to a digital data acquisition and analysis system to record heart and respiratory rates. An intravenous (IV) infusion of 4 mg NaCN/mL saline solution at a rate of 2 mL/min was initiated. Periodic blood samples were collected to measure MHb and free and total blood CN^- levels. The results of the repeated NaCN dosing of Phase I indicated that repeated dosing in Phases III, IV, VIII, and V would not impact study parameters

(TRA, baseline heart and respiratory rates). There were no apparent trends in heart rate, respiratory rate, total or free CN^- levels, or MHb levels following repeated NaCN dosing. Baseline hemoglobin levels did appear to be affected by repeated experimentation, and hematocrit readings also decreased, apparently due to the repeated blood samplings at weekly intervals.

Phases II and VII were limited pharmacodynamic experiments designed to establish a dose-MHb concentration curve over time for each test article (PAPP, WR242511, and PAHP) in this population of animals. PAPP in polyethylene glycol (PEG200) was given IV and statistical models fitted to the dose-MHb response data to estimate doses required to produce specified maximum MHb levels, the length of time to reach maximum MHb levels, and the washout time, that period required for MHb levels to return to baseline values. Estimated dose to produce 2.5, 5, and 10 percent MHb levels and the time required to reach this level are given in Table 1.

TABLE 1. ESTIMATED PAPP DOSE PRODUCING 2.5, 5 AND 10 PERCENT METHEMOGLOBIN

Percent MHb(%)	PAPP Dose[a] (mg/kg)	Time to Peak[b] (min)
2.5	0.12	28
5.0	0.17	38
10.0	0.37	56

[a] PAPP dose predicted to produce specified peak percent MHb.
[b] Predicted time to peak for specified peak percent MHb.

WR242511 was given per os in gelatin capsules and peak percent MHb levels were reached approximately 3 to 6 days after the dose was administered. After a 2.5 mg/kg dose of WR242511, the peak percent MHb level was greater than 5 percent in each animal and the MHb level remained about 5 percent for an additional 3 to 4 day period. The washout period was one week for PAPP and one month for WR242511. A pilot study determined that PAHP dissolved in PEG200 and placed in gelatin capsules could be dosed orally in Phases VII and

VIII. Thus, animals were dosed similarly to WR242511. Oral dosing of 2.0 or 5.0 mg PAHP/kg body weight (BW) in Phase VII pharmacodynamic experiments resulted in greater variability in peak percent MHb than did orally administered WR242511. This increased variability in peak MHb following oral PAHP dosing made it difficult to predict the PAHP dose-MHb level relationship.

Multiple-dose pharmacodynamic experiments were conducted in Phase VI to assess the ability of WR242511 (long-acting MHb-former) and PAHP (short-acting MHb-former) to maintain a steady state (SS) MHb level. An oral loading dose of 2 mg WR242511 crystals/kg BW was administered to each animal with subsequent dosings of 1 mg WR242511/kg BW administered every 48 hours to two weeks. PAHP was dissolved in PEG200 at a concentration sufficient to deliver the desired volume in one or two capsules. A target dose of 6.0 mg PAHP/kg BW was administered orally every 8 hours for up to nine days. The SS average was used to characterize the SS percent MHb level for WR242511, and the SS trough characterized the SS percent MHb level for PAHP. SS levels were generally reached after 4 to 6 dosings of either test compound. MHb levels following repeated dosings of WR242511 were more stable and predictable than those observed following repeated dosings of PAHP. Most animals in the WR242511 treatment group reached 95 percent of the SS percent MHb during the fourth dose period (6 to 8 days after the first dose) and stayed near that level for the remainder of the experiment. The weighted mean SS for WR242511 was 12.0 percent MHb, although the estimated SS ranged between 8.6 and 15.9 percent MHb for individual animals. For PAHP, the weighted mean SS trough was 9.4 percent MHb. While 9.4 percent represents the average minimum percent MHb during SS, the actual level was much higher at some time points. Peak levels greater than 30 percent were observed in five of the animals. To estimate a time to 95 (t_{95}) percent of SS, trough levels were modeled as an exponential function for each animal. The t_{95} ranged between 2.1 and 19.9 days with a weighted average of 2.4 days (approximately the seventh dose period).

Phase III experiments were conducted to assess the efficacy of PAPP at doses predicted to induce 0, 2.5, 5, and 10 percent MHb. Primary response parameters for PAPP efficacy evaluations were TRA, NaCN dose infused, and percent MHb. In addition, the relationships

between TRA and infused NaCN dose versus percent MHb were investigated. The following conclusions were based on the statistical analyses of the primary response parameters:

1) Average TRA (3.7, 4.4, and 9.7 min) for each PAPP-dosed group (0.12, 0.17, and 0.37 mg PAPP/kg BW, respectively) was significantly greater than the 2.4 min observed for the control group (0 mg PAPP/kg BW). A statistically significant linear relationship was observed between percent MHb and ln-transformed TRA.

2) Average amount of infused NaCN (2.3, 2.8, and 6.4 mg NaCN/kg BW, respectively) for each PAPP-dosed group was significantly greater than the 1.5 mg NaCN/kg BW observed for the control group. A statistically significant linear relationship was observed between percent MHb and ln-transformed NaCN dose infused.

3) Average percent MHb (2.9, 4.2, and 11.3 percent MHb, respectively) for each PAPP-dosed group was significantly greater than the 0 percent MHb observed for the control group.

4) Repeated dosing did not appear to have significant impact on TRA, infused NaCN dose, or percent MHb produced. The animal-to-animal variability was statistically significant for TRA and percent MHb.

The effect of PAPP dose on respiratory rate, heart rate, total blood CN^- and free plasma CN^- were also investigated. The following conclusions are based on the statistical analyses of the physiologic and NaCN response parameters:

1) Average time to peak heart rate at 0.17 and 0.37 mg PAPP/kg BW were significantly greater than that observed for the control group. The magnitude of the peak shift from baseline heart rate did not appear to be related to PAPP dose.

2) Average time to peak respiratory rate for each PAPP-dosed group was significantly greater than that observed for the control group. The magnitude of the peak shift from baseline respiratory rate did not appear to be related to PAPP dose.

3) Average total blood CN^- (13.8, 17.2, and 37.3 μg CN^-/mL, respectively) following RA for each PAPP-dosed group was significantly greater than the 6.7 μg CN^-/mL observed for the control group. The average uptake rate of total blood CN^- for each PAPP-dosed group was significantly greater than that observed for the control group.

4) Average time to peak free plasma CN^- (4.0, 4.7, and 9.7 min, respectively) for each PAPP-dosed group was significantly greater than the 2.7 min observed for the control group. Average peak levels of free CN^- (0.8, 0.6, and 0.4 μg CN^-/mL respectively) for each dose of PAPP were significantly less than the 1.3 μg/mL observed for the control group.

5) The animal-to-animal variability was statistically significant for time to peak respiratory and heart rates, peak shift from baseline levels of respiratory rate, uptake rate of total blood CN^-, and time to peak and peak levels of free plasma CN^-. Variability due to repeated dosing was statistically significant for time to peak heart rate, uptake rate of total blood CN^-, and peak levels of free CN^-.

The effect of PAPP across doses was to increase the TRA, the amount of NaCN infused until RA, and the percent MHb. In addition, the effect of PAPP dosing was to delay the initiation of increased heart rate and respiratory rate, to attenuate the rate of increase of both heart rate and respiratory rate, and prolong the time to peak heart rate, respiratory rate, TRA, and peak free CN^-. This was most dramatic when comparing the zero PAPP dose to the 0.37 mg PAPP/kg BW dose.

Phases IV and VIII were conducted to determine the efficacy of PAPP, WR242511, and PAHP, at doses producing about 5 percent MHb, in delaying cyanide-induced RA. To control animal-to-animal variability between Phase IV and Phase VIII experiments, the same animals were used in both phases. PAPP was given IV to anesthetized dogs and WR242511 and PAHP were given orally in gelatin capsules to awake dogs. The Phase IV animals pretreated with WR242511 were given 2.5 mg/kg BW and were infused with NaCN after the percent MHb levels had peaked and fallen to a range of approximately 3 to 7 percent. For PAHP, 7 mg/kg BW was administered and MHb levels were monitored until the MHb level fell within a range about 5 percent (3 to 7 percent). Once the target was reached, NaCN infusion was initiated in the anesthetized animal. When comparing Phase IV to Phase VIII, the average time from start of NaCN infusion to RA for control animals was 2.5 min in Phase IV and 2.9 min in Phase VIII. The average time from the start of NaCN infusion to RA for treated animals ranged from 5.0 min for PAPP to 5.3 min for WR242511 to 5.6 min for PAHP, while the average level of MHb when NaCN infusion began was 4.9, 5.0, and 4.9 percent, respectively. PAPP, WR242511, and PAHP pretreatment regimens were all effective in mitigating the effects of

NaCN poisoning when compared to the controls. No statistically significant differences in efficacy were observed among the three compounds. The mitigating effect appears to be related only to the induced MHb level. The relationships of the TRA and infused NaCN dose with percent MHb appear to be similar for all three compounds.

Phase V was designed to determine the pretreatment efficacy of a MHb-forming compound (PAPP) in countering the lethal effects of a standard solution of NaCN infused for twice the average time to respiratory arrest (2x AvTRA) in untreated animals. The fitted linear regression between percent MHb and infused NaCN dose determined in Phase III was utilized to predict the percent MHb required to protect against a NaCN dose twice the average dose that produced RA in dogs not pretreated. The average estimated protective percent MHb was 5.4 percent with a 95 percent upper tolerance bound of 6.4 percent. As an additional safety factor, a target MHb level of 6.5 percent was selected. Using these data, 0.3 mg PAPP/kg BW was the dose predicted to produce the approximately 6.5 percent MHb necessary to protect against a 2x AvTRA NaCN challenge. Neither hydroxylamine nor any additional therapies were necessary to ensure survival. The PAPP pretreatment regimen was effective in protecting 100 percent of the animals against a 2x AvTRA NaCN challenge. Statistically, the true proportion that would be protected under these conditions is greater than or equal to 74 percent.

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TASK 92-28:
CHARACTERIZATION OF THE ANTICYANIDE EFFECT OF METHEMOGLOBINEMIA INDUCED BY CANDIDATE PRETREATMENT DRUGS IN AN ANESTHETIZED ANIMAL MODEL

1.0 INTRODUCTION

Current therapy for cyanide (CN) intoxication consists of the IV administration of sodium nitrite and sodium thiosulfate. Field use of this treatment regimen is impractical for three reasons:

1) To be effective in counteracting CN intoxication, life saving therapy must be administered very rapidly,

2) Sodium nitrite therapy must be administered intravenously, and

3) The potential for hypotensive effects resulting from sodium nitrite administration requires that treatment be performed under medical supervision.

A preventative or prophylactic treatment regimen for CN intoxication, therefore, needs to be established to eliminate the field use constraints of current therapy. One approach is to pretreat (administer a compound prior to CN exposure) with a drug that promotes methemoglobin (MHb) formation to prevent the CN^- from binding to the cellular respiratory enzyme, cytochrome oxidase. While data exist to support the success of pretreatment therapy in various animal models, there has not been a study in which different pretreatment regimens were evaluated concurrently in the preferred canine model.

Historically, efficacy evaluations of anticyanide therapeutic compounds used lethality in unanesthetized animals as the toxic endpoint. In 1991, Vick and Froehlich developed an anesthetized canine model in which they injected an IV CN bolus and used lethality as a measure of the efficacy of MHb-forming therapeutic regimens.[1] This model was modified to utilize a slow IV infusion of CN leading to respiratory arrest (RA).[2] This modification of the Vick-Froehlich canine model replaced the classical use of lethality as the endpoint for toxicity assessment with RA for 30 seconds, and this has the advantage of permitting multiple evaluations of MHb-forming regimens in the same animal.

Objectives: Battelle's Medical Research and Evaluation Facility (MREF) conducted Task 92-28 to determine the protective efficacy provided by low levels (less than or equal to 10 percent) of MHb against a continuous NaCN infusion, and to compare and quantitate the efficacies of long-acting and short-acting MHb-forming compounds. Three MHb-forming compounds were evaluated for efficacy, and limited pharmacodynamic studies were performed by measuring MHb levels in blood. Two MHb-forming compounds were evaluated in a multiple dose study. Experiments were conducted in phases to minimize animal usage, and were not performed in numerical sequence. In this report, the phases are discussed in the following order: Feasibility Study (Phase I), Single Dose Pharmacodynamic Studies (Phases II and VII), Multiple Dose Pharmacodynamic Study (Phase VI), and Efficacy Studies (Phases III, IV, VIII and V). Individual animal data for each phase are presented in separate appendices.

Feasibility Study (Phase I): The primary objective of this phase was to determine the feasibility of repeatedly infusing NaCN into the same animal with a minimum one-week washout period. Secondary objectives were to determine the variability in time to respiratory arrest (TRA), the replicability of the procedure, and the development of trends in the data over repeated infusions.

Single Dose Pharmacodynamic Studies (Phases II and VII): The primary objective was to establish a dose-MHb concentration response over time for each test article [p-aminopropiophenone (PAPP), an 8-aminoquinoline (WR242511), and p-aminoheptanophenone (PAHP)] in this population of animals. Secondary objectives for each test article were to determine: 1) a dose that achieved an approximately 5 percent peak MHb, 2) the time required to reach peak levels of MHb, 3) the peak MHb levels reached at specific doses, and 4) the MHb washout time required to reach an approximately baseline level.

Multiple Dose Pharmacodynamic Study (Phase VI): The primary objective was to evaluate the abilities of two compounds to produce essentially constant levels (steady state) of MHb. Secondary objectives for each test article were to: 1) determine the time to reach SS levels of MHb, and the SS MHb levels reached with specific doses, 2) establish a dose-MHb concentration response curve as a function of time for the specified WR242511 and PAHP

multiple dosing regimens in this population of animals, and 3) determine for each compound the levels of MHb immediately prior to and at specified times after multiple doses.

Efficacy Studies (Phases III, IV, V, and VIII): The primary objectives for efficacy studies were to: 1) determine the protective efficacy provided by low levels (less than or equal to 10 percent) of MHb against an IV NaCN challenge, 2) compare and quantitate the efficacy of a long-acting (WR242511) and short-acting (PAPP and PAHP) MHb-forming compounds in protecting against NaCN, and 3) determine the pretreatment efficacy of a MHb-forming compound in countering the lethal effects of a solution of NaCN infused for twice the average TRA (2x AvTRA) of untreated animals (Phase V only).

2.0 MATERIALS AND METHODS

This study was conducted at Battelle's MREF following the Good Laboratory Practice (GLP) Regulations of the Food and Drug Administration (FDA). Appendix A contains MREF Protocol 98, including Addenda, Amendments, and Deviation Reports pertaining to the study. Study memorandums, contained in Appendix B, provide details on the methods and schedules used to perform each phase of the study.

2.1 Experimental Design

This task was conducted in eight phases, using the same animals in multiple phases to minimize the number of animals needed to achieve statistically valid results. Animals were assigned to succeeding phases after a washout period had elapsed. Thirty animals were accepted for use in the study. Table 2 presents a summary of the experimental design with the number of animals used in each phase. The chronological order of study performance was as follows: Phases II, I, III, IV, VII, VIII, VI, and V. Some phases were performed concurrently with others, e.g., Phases I and II, and Phases VI and VIII.

Feasibility Study (Phase I): This phase was designed to determine the variability in TRA, the replicability of the procedure, and the development of trends in the data over repeated experiments. No pretreatment MHb-forming compounds were administered during these experiments. Four animals were tested in the feasibility study.

TABLE 2. EXPERIMENTAL DESIGN SUMMARY

Phase	Description of Experiments	N
I	Repeated NaCN Dosing To Effect	4
II	Limited Methemoglobin Pharmacodynamic Studies with Two Compounds	4/compound Total 8
III	Efficacy Experiments with Four Doses of a Short-Acting Methemoglobin Former	8
IV	Comparative Efficacy Experiments with Two Methemoglobin Formers	9
V	Fixed NaCN Challenge of Dogs Given a Methemoglobin Forming Compound	10[a]
VI	Limited Multiple-Dosing Pharmacodynamic Experiments with Two Candidate Methemoglobin-Forming Compounds	6/compound Total 12[b]
VII	Limited Methemoglobin Pharmacodynamic Experiment with PAHP	6[b]
VIII	Efficacy Experiment with PAHP at Approximately 5 Percent Methemoglobin	9[a]

[a] These animals were used in Phases III or IV.
[b] Some of these animals were used in Phases I and/or II.

NaCN dosing was repeated four times in each animal, with a minimum one-week washout period between doses. Cardiac and respiratory data were collected. Blood samples were collected periodically for CN^- (total and free), Hb, and MHb analyses.

Single-Dose Pharmacodynamic Studies (Phases II and VII): Experiments for Phases II and VII were designed to establish a dose-MHb response curve over time for each test article (PAPP, WR242511, and PAHP) in this population of male beagle dogs. The dose-MHb response data were used in later phases (III, IV, VIII, and V) to estimate the test compound doses required to produce specified maximum MHb levels and to provide estimates of the length of time following dosing to attain maximum MHb levels.

Phase II (PAPP and WR242511) - For each compound, the experiment used a four-period, cross-over design, with four animals and four doses as shown in Table 3. Each period contained the four doses of the test compound being evaluated and each animal received each of the four doses with a washout period between each dose. Limited data provided by U.S. Army personnel were used to estimate compound doses necessary to attain target MHb levels. Table 4 lists PAPP and WR242511 doses used in Phase II along with targeted MHb

levels. Blood samples were collected periodically for Hb and MHb analyses. Cardiac and respiratory data were not collected. The washout period was one week for PAPP and one month for WR242511.

TABLE 3. PHASE II-EXPERIMENTAL DESIGN[a]

	Period			
Animal	1	2	3	4
1	A	B	C	D
2	B	C	D	A
3	D	A	B	C
4	C	D	A	B

[a] Doses to produce MHb - A ≈ 2.5%, B ≈ 5%, C ≈ 10%, D ≈ 15%

TABLE 4. PHASE II-TARGET DOSES OF PAPP AND WR242511 FOR PHARMACODYNAMIC EXPERIMENTS

Dose	Target MHb (%)	Target PAPP Dose (mg/kg)	Target WR242511 Dose (mg/kg)
A	2.5	0.10	1.3
B	5.0	0.13	2.5
C	10.0	0.22	5.0
D	15.0	0.37	7.4

Phase VII (PAHP) - A pilot study was performed as the initial portion of Phase VII to determine whether PAHP could be administered per os in a gelatin capsule. The pilot study of Phase VII and results are reported in Appendix F. A limited pharmacodynamic experiment followed the pilot study, and was designed to establish a PAHP dose-MHb response curve over time in this population of male beagle dogs. The PAHP pharmacodynamic experiment was a two period cross-over design, using six animals randomly selected from those animals used in

Phase II and two compound doses as shown in Table 5. In each period, each of the two doses of PAHP (2.0 and 5.0 mg/kg BW) was given to three animals. Doses were reversed for the second period, so that each animal received each of the two doses with a one-week washout period between doses. Animals were randomly assigned to treatment sequence. Periodic blood samples were collected for Hb and MHb analyses. Cardiac and respiratory data were not collected. In each period, all six animals were dosed in one day by staggering dosing and bleeding times.

TABLE 5. PHASE VII-EXPERIMENTAL DESIGN

Animal	Dose of PAHP(mg/kg) Given in Period	
	1	2
HFXAGU	2.0	5.0
HFYBEI	5.0	2.0
HFZAYM	2.0	5.0
HFXBGI	5.0	2.0
HFYBCP	2.0	5.0
HFZBFI	5.0	2.0

Multiple Dose Pharmacodynamic Study (Phase VI): This phase was conducted to determine whether a SS MHb level could be maintained over time using either of two MHb-forming compounds (WR242511 or PAHP). The animals used in Phase VI PAHP and WR242511 experiments had been used previously in Phases I and II. A minimum three month washout period was allowed in all animals prior to Phase VI. Blood samples were collected for Hb and MHb analyses. Cardiac and respiratory data were not collected. For the WR242511 experiment, a loading dose of 2 mg/kg BW was administered per os to each of six animals using gelatin capsules. Subsequent doses were 1 mg/kg administered every 48 hr for two weeks. For the PAHP experiment, 6 mg/kg BW was given in gelatin capsules to each of six animals every 8 hr for up to nine days.

Efficacy Studies (Phases III, IV, V and VIII): Phase III (PAPP) - This phase was designed to determine the efficacy of a short-acting, MHb-forming compound, at doses expected to induce MHb levels of 2.5, 5, or 10 percent, in delaying CN-induced RA. The experiment was a four-period, cross-over design, using a total of eight animals in a randomized block, as shown in Table 6. During each period of Phase III testing, each of the four doses was administered to two animals. A single animal was dosed each day a study was performed. Thus, after four periods, each animal had received each dose with an approximately two-week washout period between dosings. PAPP doses and timing of NaCN infusion, presented in Table 7, were based on Phase II results. PAPP doses were selected to produce MHb levels that peaked at or slightly above the targeted level. NaCN infusion was started at the time the targeted MHb level was predicted to be reached. Cardiac and respiratory data were collected. Blood samples were collected periodically for CN^- level (total and free), and Hb and MHb analyses.

TABLE 6. PHASE III-EXPERIMENTAL DESIGN[a]

Animal	Period			
	1	2	3	4
1	A	B	D	C
2	B	C	A	D
3	C	D	B	A
4	D	A	C	B
5	A	B	D	C
6	B	C	A	D
7	C	D	B	A
8	D	A	C	B

[a] Targeted Doses - A ≈ 0%, B ≈ 2.5%, C ≈ 5%, D ≈ 10% MHb

TABLE 7. PHASE III-PAPP DOSES AND TIMING OF CYANIDE INFUSION

Dose	Targeted MHb (%)	PAPP Dose (mg/kg)	NaCN Infusion Start (min post PAPP dosing)
A	0.0	0.0	28
B	2.5	0.12	28
C	5.0	0.17	37
D	10.0	0.37	56

Phase IV (PAPP and WR242511) and Phase VIII (PAHP) - Phases IV and VIII were designed to compare the efficacy of three MHb-forming compounds, given at doses to produce similar MHb levels, in delaying CN-induced RA. Initially, two MHb-forming compounds were specified for evaluation; a third MHb-forming compound (Phase VIII) was added. In Phase IV, nine animals were used in the three period experimental design shown in Table 8. The test compound subscripts "s" and "l" indicate the short-acting (PAPP) and long-acting, (WR242511) MHb-forming compounds, respectively. While it would have been ideal to randomize dosing of the short-acting and long-acting, MHb-forming compounds over the second and third periods, the long biological half-life of WR242511 precluded this approach. Therefore, the two MHb-forming compounds were examined in a systematic fashion. Generally, two animals per day were infused with NaCN with a minimum one-week washout between each period. Doses and administration technique for PAPP were based on those used in Phase III. The MHb levels produced by PAPP were those targeted for the orally-administered WR242511. Each animal served as its own control. Cardiac and respiratory data were collected. Blood samples were collected periodically for total CN^- concentration, and Hb and MHb analyses.

Phase VIII - This phase was designed to test the efficacy of a second short-acting, MHb-forming compound (PAHP) in delaying CN-induced RA at a MHb level similar to that targeted in Phase IV experiments. PAHP was added for evaluation following an approximately one-month washout of Phase IV animals. A pilot study initiated Phase VIII to determine if the dosage of PAHP selected produced a MHb level of about 5 percent. A detailed description of the Phase VIII pilot study with data and results are in Appendix J and Study Memorandums dated June 14 and June 17, 1994 in Appendix B. The efficacy experiment used the two-period, cross-over design shown in Table 9. Nine animals previously used in Phase IV were used again in Phase VIII. Each animal served as its own control. Two animals per day were infused with a NaCN solution with a minimum one-week washout between periods. On each dosing day, one animal received PAHP pretreatment and the other the vehicle control. Doses and administration technique for PAHP were based on the results of pharmacodynamic experiments conducted in Phase VII and in the Phase VIII pilot study described in Appendix J.

Cardiac and respiratory data were collected. Blood samples were collected periodically for total CN^- concentration, and Hb and MHb analyses.

Phase V - This phase was designed to determine the efficacy of a MHb-forming compound administered prior to NaCN in countering the lethal effects of NaCN administered at 2x AvTRA. Phases I, III and IV results were used to estimate the 2x AvTRA NaCN dose

TABLE 8. PHASE IV-EXPERIMENTAL DESIGN

	Period		
Animal	1	2	3
1	A[a]	B_s[b]	B_l[c]
2	A	B_s	B_l
3	A	B_s	B_l
4	A	B_s	B_l
5	A	B_s	B_l
6	A	B_s	B_l
7	A	B_s	B_l
8	A	B_s	B_l
9	A	B_s	B_l

[a] Dose A - = 0 mg/kg; PEG200 as vehicle control
[b] Dose B_s - ≃ 0.2 mg PAPP/kg BW, expected to produce ≃ 5 percent MHb
[c] Dose B_l - ≃ 2.5 mg WR242511/kg BW, expected to produce greater than 5 percent MHb

TABLE 9. PHASE VIII-EXPERIMENTAL DESIGN

	Period	
Animal	1	2
1	B[a]	A[b]
2	A	B
3	B	A
4	A	B
5	B	A
6	A	B
7	B	A
8	A	B
9	B	A

[a] Dose B of PAHP - ≃ 7 mg PAHP/kg BW per os; expected to produce ≃ 5 percent MHb.
[b] Dose A of PAHP - 0 mg; PEG200 as vehicle control.

and the MHb level needed to protect against a 2x AvTRA NaCN challenge. Phase II results were used to select a PAPP dose that would result in at least that level of MHb predicted to protect against a 2x AvTRA NaCN dose. Phase V was a single-period experiment using ten animals randomly selected from a group of 17 animals previously used in other phases. The minimum washout period between phases was one month. Two animals per day were pretreated with 0.3 mg PAPP/kg BW and infused with a 2x AvTRA NaCN challenge. The primary endpoint was survival. In addition, cardiac and respiratory data were collected and analyzed. Blood samples were collected periodically for total CN^- concentration, and Hb and MHb analyses.

2.2. Test System

Male Beagle dogs, *Canis familiaris*, were specified for use in this study. The dog is the preferred animal model for anti-CN, MHb-inducing drug testing as the enzymatic profiles and kinetics of MHb formation are similar for dog and man.[3] There is considerable scientific evidence that canine MHb reductase activity is similar to that of man, and therefore predictive of MHb responses in man.[4,5,6,7] The MHb reductase activity in rodents is significantly greater than that of man.[8,9] Lacking glucuronyl transferase, felines do not conjugate phenols to glucuronic acid, resulting in the inability to metabolize many of the MHb-forming drugs.[10] Swine have been recommended as an appropriate model; however, their primary energy source for MHb reduction is lactate as opposed to glucose in dogs and humans.[11] Since CN intoxication produces a severe lactate acidosis[12], further development of an *in vivo* swine model will depend upon the impact of the different energy source on MHb reduction in the two species. Dogs have been historically used as the animal model of choice for studying CN therapy and an extensive data base exists.[3] Other animal models currently are not available for such evaluations because of differences in enzymatic profiles and kinetics of MHb formation between man and other species.[3,8,9,10,11,12] The United States Army Medical Research and Materiel Command (USAMRMC) has selected the dog for use in their Decision Tree Network for CN intoxication treatment studies. Additionally, the dog was recommended as the preferred animal model by North Atlantic Treaty Organization scientists in the

December 1991 findings of the Research Study Group-3 Panel on CN Research. The extensive canine data base also contains information on MHb formation/reduction kinetics for several 8-aminoquinolines proposed as MHb-forming, CN^- intoxication treatment regimens.

Thirty-six male beagles from HRP Incorporated (Kalamazoo, MI) were received at Battelle on May 28, 1993. Dogs weighed a minimum of 8 kg upon receipt at the MREF and were approximately 6 months of age. Dogs generally were individually housed during quarantine, maintenance, and study periods. Individual stainless-steel cages were used to house the dogs. Housing complied with the 1991 revised Animal Welfare Act. Fluorescent lighting was used with a light/dark cycle of approximately 12 hr each per day. The air temperature in animal holding rooms was maintained, as possible, between 65 and 84 degrees F. Relative humidity in rooms was maintained, as possible, at 30-70 percent. At least 95 percent of the total recordings for air temperature and relative humidity fell within the specified ranges.

Purina certified dog chow (PMI Mills, Richmond, IN) was fed twice daily. Animals that were not eating well, or needed additional supplementation, received canned dog food in addition to the chow. Canned dog food was fed as needed. Water was supplied *ad libitum* using an Edstrom automatic watering system (Edstrom Industries, Inc., Waterford, WI) or by water bowl. Water is analyzed annually for potability and for contaminants. No contaminants that would interfere with the results of the study are known to be present in the feed or water.

Animals were received with ear tattoos so that positive identification could be maintained. Blood samples were collected for hematology and clinical chemistry analyses, and fecal samples were analyzed for gastrointestinal parasite infestation during the quarantine period. No significant abnormalities were noted. Physicals during quarantine indicated that a majority of the dogs displayed a mild otitis externa consistent with an overgrowth of *Malassezia canis*, a yeast organism normally found in the canine ear canal. The animals were treated with Panalog® ointment (twice a day for 8 days) and cleaning of the ear canal (once a day during the 8 days). Animal HFYBDE was observed to seizure and a physical examination revealed that animal HFYAVZ had a systolic heart murmur. These animals were replaced with animals HFYAGL and HFZAAD, received at Battelle on June 15, 1993. Animals were released from quarantine on July 1, 1993 and the first 4 animals were placed on study

beginning September 29, 1993. During the period that animals were maintained by Battelle, occasional minor medical problems were noted and treated as necessary. Minor medical problems included periodic bouts of otitis externa or conjunctivitis, interdigital cysts, occasional emesis and/or diarrhea, a torn nail, and minor cuts. Animal HFYAWM was diagnosed with "cherry eye" (protrusion of the nictitans gland of the third eyelid) with secondary conjunctivitis in the left eye. The nictitans glands was surgically removed, but a portion of the gland remained and the animal displayed periodic conjunctivitis. HFZAGX had an umbilical hernia which was surgically repaired. None of the health problems mentioned appeared to have had any adverse effects on the various parameters measured nor were known to interfere with study results. Most health problems occurred when animals were not actively involved in a study phase.

Discomfort and injury of animals was limited to that which was unavoidable in the conduct of scientifically valuable research. Animals were anesthetized during NaCN infusion to minimize any discomfort, injury, or anxiety. Animals were closely monitored throughout the study for signs of discomfort, such as anorexia, dehydration, excessive weight loss, lethargy, or prostration. If, in the opinion of a Battelle veterinarian, a dog appeared to be suffering or in a moribund state, that animal would have been euthanatized with an approved euthanasia solution. No animal exhibited signs of discomfort, nor was in a moribund state as a result of this task. Only one animal, HFXBDB, died on study. This occurred early in Phase II (second week), apparently due either to an adverse reaction to the anesthetic or possibly an anesthetic overdose, although the animal received less than the dose calculated to be necessary. This dog was replaced by animal HFXBGH.

Initial individual body weights measured during MREF acclimation were used to assign animals to treatment groups. Phases I, II, III, and IV used naive animals, while animals used in Phase V were randomly selected from those used previously in Phases III and IV. Animals used in Phase VI were randomly selected from those used previously in Phases I and II. Animals used in Phase VII were randomly selected from those used previously in Phase II. Phase VIII animals were those used in Phase IV. Table 10 is a list of the animals placed on study, the phases to which they were assigned, and their disposition.

The 37 surviving animals (including the two replacement animals) used in Task 92-28 were donated to other laboratories, either Battelle Columbus or The Ohio State University (OSU). The sponsor submitted a memorandum releasing the animals from study on September 21, 1994. With permission of the sponsor, the animals were given either to OSU (34 animals) or to another research group within Battelle (3 animals).

TABLE 10. SUMMARY OF ANIMAL STUDY EXPOSURES AND DISPOSITION

		Disposition
1.	HFXAYH - Phase I, Phase VI	OSU
2.	HFZADI - Phase I, Phase VI	OSU
3.	HFXAYK - Phase I, Phase VI	OSU
4.	HFZADP - Phase I, Phase VI	OSU
5.	HFZAYM - Phase II, Phase VI, Phase VII	OSU
6.	HFXBDB - Phase II - died early in Phase II	Died
7.	HFXBGH - Phase II - replaced HFXBDB, Phase VI	OSU
8.	HFXBAD - Phase II, Phase VI	OSU
9.	HFXAGU - Phase II, Phase VI, Phase VII, Pilot Phase VIII	OSU
10.	HFZBFI - Phase II, Phase VI, Phase VII	OSU
11.	HFYBEI - Phase II, Phase VI, Phase VII, Pilot Phase VIII	OSU
12.	HFYBCP - Phase II, Phase VI, Phase VII	OSU
13.	HFXBGI - Phase II, Phase VI, Phase VII	OSU
14.	HFZADG - Phase III, Phase V	OSU
15.	HFZADD - Phase III, Phase V	OSU
16.	HFYAZF - Phase III	OSU
17.	HFYAIH - Phase III	OSU
18.	HFZBDC - Phase III, Phase V	OSU
19.	HFXAEN - Phase III, Phase V, Phase VII Pilot	OSU
20.	HFYBKC - Phase III, Phase V	OSU
21.	HFYBFM - Phase III, Phase V, Phase VII Pilot	OSU
22.	HFYAGL - Phase IV, Phase VIII	OSU
23.	HFZANH - Phase IV, Phase VIII	OSU
24.	HFYAWJ - Phase IV, Phase VIII, Phase V	OSU
25.	HFZAGX - Phase IV, Phase VIII, Phase V	OSU
26.	HFYAJM - Phase IV, Phase VIII, Phase V	OSU
27.	HFZAHB - Phase IV, Phase VIII	OSU
28.	HFYATS - Phase IV, Phase VIII	OSU
29.	HFYBJC - Phase IV, Phase VIII	OSU
30.	HFZBAV - Phase IV, Phase VIII, Phase V	OSU
31.	HFZAAD - Extra	OSU
32.	HFZAYK - Extra	Battelle
33.	HFYAWM - Extra	OSU
34.	HFXBER - Extra	OSU
35.	HFZAHI - Extra	Battelle
36.	HFZAVG - Extra	Battelle
37.	HFYAVZ - Systolic Heart Murmur - Replaced*	OSU
38.	HFYBDE - Seizure - Replaced*	OSU

* Animals replaced during quarantine.

2.3. Chemistry

A solution preparation data sheet was developed by chemistry personnel with instructions for the preparation of each of the following: 4.0 mg/mL NaCN infusion solution, 50.0 mg/mL hydroxylamine hydrochloride solution, 10.0 mg/mL methylene blue solution, 250 mg/mL sodium thiosulfate solution, and 5.0 mg/mL atropine sulfate solution (See Appendix C, Attachment C.I for these instructions.). Dose solution concentrations were confirmed for NaCN infusion solutions, hydroxylamine hydrochloride solution, and the test articles PAPP and PAHP. A complete report of results and methodologies for these analyses are given in Appendix C.

Test Toxicant - Sodium Cyanide (NaCN): NaCN, Lot Number 07723JX, was purchased from Aldrich Chemical Co. (Milwaukee, WI), received July 22, 1993, and stored in a desiccator in the medical safe at room temperature. For Phases I, III, IV, and VIII, a NaCN infusion solution with a target concentration of 4.0 mg/mL was prepared daily according to the method described in Appendix C. For Phase V, a stock of an approximately 4.0 mg/mL NaCN infusion solution was prepared and divided into dosing aliquots that were analyzed on the day of infusion. Dose confirmation samples of NaCN infusion solutions were analyzed using the total cyanide process, an automated microdistillation cyanide assay, performed on the Technicon AutoAnalyzer II®. Procedures for analysis of both free plasma and total cyanide ion in a sample matrix, validation of these processes, and results are included in Appendix C. The results of CN^- concentration analyses indicated that the values ranged from 91.2 to 98.5 percent of expected over a five-day validation period. Control NaCN samples were prepared at 0.060, 0.100, 0.220, and 0.400 mM. These control samples were analyzed over the 80 days of the infusion test period. Results (Figures C.1.2 through C.1.5) of these analyses indicate that the relative error for each control sample was 0.0, 2.0, 0.5 and 0.8 percent, respectively.

Test Articles: Identity, composition, purity and stability analyses of the test articles were the responsibility of the USAMRMC, and were not duplicated by Battelle. Test article dosing solution concentrations (PAPP and PAHP) and identity (PAPP, PAHP, WR242511) were confirmed by Battelle.

WR302 (PAPP): A short-acting MHb-forming test article, para-aminopropiophenone, Lot number BM11449 (received July 15, 1993), was provided by the Walter Reed Army Institute of Research (WRAIR) and stored in a refrigerator at approximately 0 to 10 C. Identity, purity, and additional chemical analyses of PAPP had been performed for the U.S. Army by SRI International under Contract No. DAMD17-91-C-1135, Project No. 2653, and results provided in Report No. 768, dated July 13, 1992. PAPP dosing solutions were prepared on each day of use at various concentrations depending upon each animal's body weight. For a specific concentration, the appropriate amount of PAPP was weighed, placed into a 10-mL volumetric flask and the diluent, PEG200, added. Identity verification and concentration confirmation of PAPP were accomplished by high performance liquid chromatography (HPLC) as reported in Appendix C, Section C.2. Table C.2.1 in Appendix C gives the PAPP concentration analysis results. Analysis of PAPP dosing solutions indicated that concentrations were within 10 percent of target values.

WR242511: The long-acting MHb-forming test article, 8-[(4-amino-1-methylbutyl)amino]-5-(1-hexyloxy)-6-methoxy-4-methylquinoline DL-tartrate (WR242511), Lot Number BM05816 (received July 15, 1993) was provided by WRAIR and stored at 0 to 10 C. Identity, purity, and additional chemical analyses had been performed by SRI International under U.S. Army Contract No. DAMD17-91-C-1135, Project No. 2653, and provided in Report No. 738, Addendum 1 to Report No. 720, dated August 7, 1991. Identity confirmation for WR242511 was accomplished by HPLC analysis. The SRI report and HPLC chromatograms are in Appendix C, Attachment C.V. Dosing capsules of WR242511 were prepared on the day of use in Phases II and IV. The appropriate amount of crystalline WR242511, calculated using the body weight of each animal, was weighed and placed into a gelatin capsule. For Phase VI, multiple capsules were prepared prior to dosing and stored at -10 to 10 C.

WR269410 (PAHP): The short-acting MHb-forming test article, para-aminoheptanophenone (WR269410 or PAHP), Lot Numbers BM08586 (for identity and standard preparation, received April 28, 1994 and May 3, 1994) and BM11565 (for dosing stock solutions, received May 27 and July 15, 1994), was provided by WRAIR and stored at

0 to 10 C. Identity, purity, and additional chemical analyses of PAHP had been performed by SRI International under Contract No. DAMD17-91-C-1135, Project No. 2653, and results provided in Report No. 770, dated June 16, 1992. During Phases VII and VIII, an approximately 120 mg PAHP/mL in PEG200 stock solution was prepared. Each animal's body weight was used to calculate the volume of diluted stock solution that was added by weight to gelatin capsules for each dog. Capsules were prepared just prior to dosing. A limited stability study of the PAHP stock solution was initiated during Phase VII. For a specific final concentration for capsule preparation, the appropriate volume of PAHP stock solution was pipetted into a 10-mL volumetric flask and PEG200 added. In Phase VI, an approximately 124 mg/mL in PEG200 stock solution was formulated for capsule preparation. Identity verification and concentration confirmation of PAHP (BM11565 or BM08586) were performed by HPLC using the same method as described for PAPP in Appendix C, Section C.2. Table C-4 in Appendix C presents the PAHP concentration analysis results. Chemical analyses of PAHP dosing stock solutions indicated that concentrations were within 10 percent of target values.

Therapeutic Compounds: Additional therapies for cyanide intoxication were prepared for administration only when necessary to ensure survival. Hydroxylamine is a very rapid MHb-forming compound and was prepared to be administered to each animal following 30 sec of CN-induced RA. Methylene blue was prepared to give IV when MHb was greater than 50 percent. Sodium thiosulfate provides additional sulfane sulfur groups to combine with cyanide to form non-toxic thiocyanate, which can be excreted by the kidney. Atropine sulfate can be administered to block vagal slowing of the heart, and lidocaine hydrochloride can be used to decrease ventricular arrhythmias.

Hydroxylamine: Hydroxylamine, Lot/Catalog Number 15.941-7, from Aldrich Chemical Co. was provided by WRAIR on June 9, 1993, and stored at room temperature. For each phase requiring hydroxylamine (Phases I, III, IV, VIII, and V), dosing aliquots were prepared prior to initiating the phase. A 50 mg/mL sterile saline solution was prepared in accordance with instructions provided in Appendix C, Attachment C.I, and stored at room

temperature. Approximately 15 mL of each day's dosing aliquot were analyzed by titration as outlined in Appendix C, Section C.3. Results for these analyses are given in Table C-5.

Methylene Blue: Methylene blue, Lot number AJ31899 X035, 1 5645, from Hartman Leddon was provided by WRAIR on June 9, 1993. For each phase requiring NaCN infusion, aliquots for each dosing day were prepared prior to initiating the phase. A 10.0 mg/mL sterile saline solution was prepared in accordance with instructions given in Appendix C, and stored in the medical safe at room temperature. Identity, stability, purity, and concentration analyses were not performed on this compound. This compound was administered twice during Phase III to animals with MHb levels greater than 50 percent.

Sodium Thiosulfate: Sodium thiosulfate, Lot number AJ00563, from General Chemical was provided by WRAIR on June 9, 1993. For each phase requiring NaCN infusion, dosing aliquots were prepared prior to initiating the phase. A 250 mg/mL sterile saline solution was prepared in dosing aliquots according to the instructions of Appendix C, Attachment C.1., and stored in the medical safe at room temperature. Identity, stability, purity, and concentration analyses were not performed on this compound. It was never necessary to administer this compound.

Atropine Sulfate: Atropine sulfate, Lot number 41H0934, was purchased commercially (Butler, Columbus, OH). A 5.0 mg/mL sterile saline solution was prepared in dosing aliquots according to the instructions in Appendix C. Dosing aliquots were stored in the medical safe at room temperature. Identity, stability, purity, and concentration analyses were not performed on this compound. Atropine sulfate was administered once in Phase I and three times in Phase III for severe bradycardia.

Lidocaine Hydrochloride: Lidocaine hydrochloride (20 mg/mL) was purchased commercially (Prolabs Limited, St. Joseph, MO) and stored in the medical safe at room temperature. Identity, stability, purity, and concentration analyses were not performed on this compound. Lidocaine hydrochloride was administered three times in Phase III and once in Phase IV to treat ventricular arrhythmias.

Anesthetic Compounds: Anesthesia Induction: The ultra-short-acting barbiturate used in Phases I and II, and in Phase III through February 21, 1994 was thiamylal sodium (Surital®;

20 mg/mL) manufactured by Parke-Davis (Parke-Davis, Morris Plains, NJ). During Phase III, thiamylal sodium became commercially unavailable. Thiopental sodium (Pentothal®), an equivalent ultra-short-acting barbiturate manufactured by Abbott Laboratories (Chicago, IL), was purchased. No apparent changes in the physiological end-points monitored were observed as a result of this change. Through the remainder of this study, thiopental sodium (25 mg/mL) was given to effect to induce anesthesia. Powdered ultra-short-acting barbiturate was reconstituted to the desired concentration using sterile water for injection per the manufacturer's directions. Surital® was stored as appropriate and a new bottle was reconstituted on a weekly basis as needed. Reconstituted Pentothal® was stored as appropriate and used within 48 hr. Identity, stability, purity, and concentration analyses were not performed on these compounds.

Anesthesia: A pentobarbital sodium solution (64.8 mg/mL) manufactured by Fort Dodge (Fort Dodge, IA) was purchased from W.A. Butler (Columbus, OH), and was used to maintain anesthesia. Pentobarbital was stored in the medical safe at room temperature. Identity, stability, purity, and concentration analyses were not performed on this compound.

2.4. Experimental Methods

The experimental methods and procedures are described in detail for Phase I. Experimental methods and procedures described for subsequent phases are only those different than, or additional to, those used in Phase I.

Feasibility Study (Phase I): The following procedures were the standard protocol for the day prior to NaCN infusion and the day of NaCN infusion.

Day Prior to NaCN Infusion: Each animal's weight and hematocrit were measured within 24 hr of study initiation. The hematocrit readings were taken to detect the development of anemia. The animals were clipped of hair for placement of catheters and electrocardiogram (ECG) leads (PO-NE-MAH Inc., Simsbury, CT). Feed was removed approximately 18 hr prior to NaCN infusion and returned upon recovery from anesthesia. Water was given *ad libitum*.

Day of NaCN Infusion: Catheter placement sites were swabbed with a Betadine Surgical Scrub solution (0.75 percent, Purdue Frederick Co., Norwalk, CT) prior to catheter placement. Generally, the left cephalic vein was catheterized with a 1.25-in, 20-ga Abbocath® (Abbott Laboratories, Chicago, IL) for anesthetic and treatment administration. Anesthesia was induced with an ultra-short-acting barbiturate (either approximately 5 mg/lb of thiamylal sodium or 7 mg/lb of thiopental sodium). The short-acting barbiturate, sodium pentobarbital, was administered to effect to maintain anesthesia. Once anesthetized, each animal was intubated with an appropriate size (7 to 8 mm internal diameter) Matrix Endotracheal Tube (J.A. Webster, Sterling, MA). One-inch gauze was used to secure the endotracheal tube to the muzzle.

Other catheters were placed for blood collection, therapeutic compound administration, and NaCN infusion. A 19-ga, 8-in Deseret Intracath® (Baxter Scientific Products, McGaw Park, IL) was inserted, generally in the right jugular vein, for blood collection. A 20-ga, 1.25-in Abbocath® was placed in the saphenous vein of the rear leg on the side opposite to the placement of the jugular catheter. This saphenous catheter was used to infuse NaCN and to administer hydroxylamine followed by an approximately 5 mL saline flush. Catheters were secured in place with surgical tape and/or Vetwrap™ (3-M Company, 3-M Animal Care Products, St. Paul, MN). Any deviations from these catheter placements were noted in the study file. Heparinized (10,000 units/mL heparin diluted to 2 units heparin/mL saline solution, Elkins-Sinn, Inc., Cherry Hill, NJ) saline blocks were used to maintain patency of the blood collection catheter and were removed prior to blood collection. Sterile three-way stopcocks (Baxter, Baxter Healthcare Corporation, Pharmaseal Division, Valencia, CA) with male leur-lock adapters were attached to the catheters. If additional IV therapies were needed, administration was through either the cephalic or saphenous catheter.

Following catheterizations, animals were placed on a circulating warm-water heating pad (J.A. Webster) and instrumented. Instrumentation consisted of connections to the PO-NE-MAH® Digital Data Acquisition, Analysis, and Archiving System (PO-NE-MAH, Inc., Simsbury, CT) to monitor heart rate and respiratory parameters. Heart rate was monitored using an ECG with leads attached to each limb and a fifth lead as a ground. The

respiratory parameters were monitored using a Fliesch Pneumotachometer (PO-NE-MAH, Inc., Simsbury, CT) attached to the endotracheal tube. Manual evaluations and calculations of chart recordings were used at specified events and/or data collection time points. Using both methods served as a systems check and as part of the validation of the PO-NE-MAH® system. After instrumentation, animals were given at least 5 min for recordings to stabilize prior to NaCN infusion.

For infusions in Phase I, a 30-mL syringe was used to draw up approximately 24 mL of the NaCN in saline solution. The hub of the syringe was connected to a 20-in Medex-Inc-MX45O-FL extension tube (Medex Inc., Hilliard, OH) and the loaded syringe with extension tube was weighed on a calibrated Mettler balance (American Scientific Products, Inc., Columbus, OH) prior to infusing NaCN. The syringe and extension tube with the remainder of the NaCN solution were reweighed after infusion and the weight difference used to calculate the amount of NaCN administered. An approximately 4 mg/mL NaCN in saline solution was infused (Harvard 44 Programmable Syringe Pump, Harvard Apparatus, South Natick, MA) continuously at a rate of approximately 2 mL/min into the catheterized saphenous vein until RA. Once RA was observed, a stop-watch was started. Cyanide infusion continued until 10 seconds had elapsed with no observable, functional breaths. Thirty sec after CN infusion was stopped, hydroxylamine (10 mg/kg) was injected into the catheterized saphenous vein. An appropriate size syringe was used to administer hydroxylamine to produce MHb for reversal of the CN toxicity. Approximately 5 mL of saline was used to flush the hydroxylamine into the animal. Respiratory assistance and other symptomatic therapies were given as needed to ensure survival of the animal.

Blood samples were collected periodically to measure Hb, MHb, and free and total CN^-. Blood samples were drawn from the jugular catheter using either a 3-mL or 1-mL heparinized (1,000 units/mL heparin solution was used to coat the inside of blood collection syringes) syringe. Hb and MHb were analyzed as soon as possible using an OSM^3 Hemoximeter® (Radiometer of America, Inc., Westlake, OH). A fluorometric assay for the determination of free and total CN^- in whole blood was developed for use on the Technicon® AA II (Technicon Instruments Corporation, Tarrytown, NJ) supplied by the U.S.

Army Medical Research Institute of Chemical Defense, (USAMRICD, Aberdeen Proving Ground, MD). This analysis method is based on the microdistillation assay of Groff, et al.[13] The presence of CN^- in a sample results in the formation of an 8-hydroxy-5-quinoline sulfonic acid chelate, which is fluorometrically detected and recorded as a peak on a strip chart.

Figure 1 is a typical tracing of a pulmonary response from which measurements of TRA were obtained during the Phase I experiment. The primary responses measured were TRA, percent MHb, and amount of NaCN infused. Secondary responses measured included heart rate, respiratory rate, Hb levels, and total and free CN^- levels in whole blood.

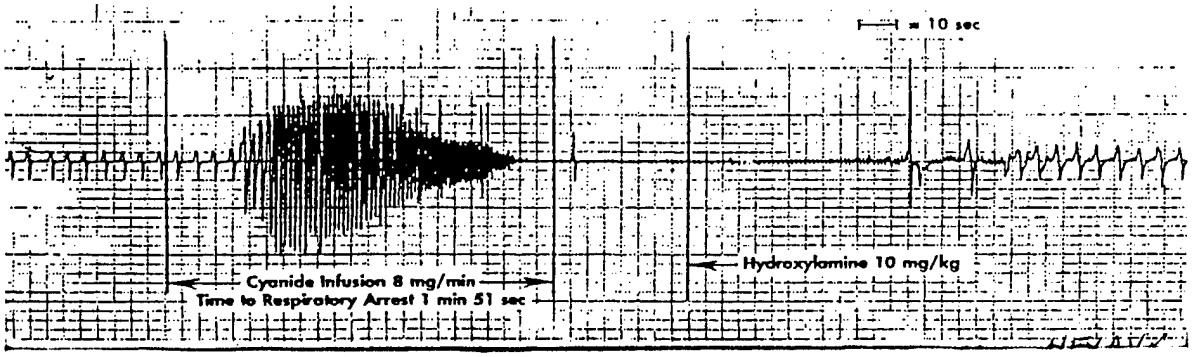

FIGURE 1. PHASE I-TYPICAL PULMONARY TRACING FROM DOG HFXAYK DURING THE CYANIDE INFUSION

Single Dose Pharmacodynamic Studies (Phases II and VII): Phase II - Animal preparation for study initiation, catheter placement, and anesthesia procedures were the same as those described for Phase I, except as described below. Blood CN^- concentration and cardiac and respiratory data were not collected. Food was returned to PAPP-dosed animals when recovery from anesthesia was complete. Food was returned to the unanesthetized WR242511-dosed animals approximately 2 hr after dosing. Animals were monitored for at least 0.5 hr after capsule administration to be sure the capsules were not regurgitated.

PAPP Administration: Animals receiving PAPP were anesthetized with thiamylal sodium and anesthesia maintained with pentobarbital sodium. Dogs were catheterized as described for Phase I. Following catheterizations, each anesthetized animal was placed on a circulating warm-water heating pad and moved to the study room. PAPP was formulated in PEG200 (Sigma Chemical Co., St. Louis, MO) for a 1-min manual infusion using the saphenous vein. Each of the four animals was given doses of 0.10, 0.13, 0.22, and 0.37 mg

PAPP/kg BW, dosing one or two animals per day with a minimum one-week washout period between dosings. Usually, a 3-mL syringe attached to a 21-ga, 1-in needle was used to administer PAPP in the saphenous vein on the side opposite that used for blood collection. After the first period of dosing, a 20-ga, 1.25-in Abbocath® was placed in the saphenous vein of the side opposite the jugular catheter.

Approximately 0.5-mL blood samples were drawn (as described in Appendix B, Study Memorandums dated September 27, and October 4, 13, and 28, 1993) from a catheterized jugular vein, using a 1-mL heparinized syringe. Blood samples were analyzed for Hb and MHb. In general, blood sampling was stopped when the MHb level returned to below 2 percent.

WR242511 Administration: Animals receiving WR242511 were neither anesthetized, catheterized, nor instrumented for data collection. The four animals were tested at doses of 1.3, 2.5, 5.0, and 7.4 mg WR242511/kg BW using gelatin capsules (No. OO, Eli Lilly and Company, Indianapolis, IN) administered orally. In this case, all four animals were dosed on the same day by staggering dosing and bleeding times (see Appendix B, Study Memorandums of September 27, October 4 and 28, and November 8 and 29, 1993). On the Period I dosing day, animal HFZBFI vomited 1 hr and 50 min after dosing, but there was no evidence of the capsule in the vomitus. On the Period 3 dosing day, animal HFYBCP regurgitated the capsule immediately after administration. The capsule was recovered and re-administered within 30 sec of the initial dosing. Blood sample draws were alternated between saphenous and cephalic veins, as necessary, with efforts made to maintain consistency in procedures. Blood samples were analyzed for Hb and MHb. The washout time between dosings was a minimum of 4 weeks, or until the MHb levels of all dogs had returned essentially to baseline.

Phase VII - A pilot study was conducted initially, using two dogs previously studied in Phase III, to compare the bioavailability of crystalline PAHP and PAHP in PEG200 given orally in gelatin capsules. This pilot study was followed by a limited pharmacodynamic study designed to estimate a PAHP dose-MHb response curve over time in this population of dogs using PAHP dissolved in PEG200 and dosed in gelatin capsule. Six dogs previously used in Phase II were randomly assigned to treatment groups. Animal preparation and PAHP dosing

procedures were essentially the same as described for Phases I and II, the Phase VII Pilot Study (See Appendix F, Phase VII Pilot Study), and Study Memorandums dated May 6 and 18, 1994 (Appendix B). Periodic blood samples were collected and analyzed as described in the Study Memorandum of May 6, 1994 in Appendix B. All six animals were dosed in one day by staggering the dosing and bleeding times.

For animals whose MHb did not reach five percent, the bleeding schedule was changed from every 15 min to every 30 min. Blood sampling for dosing-day pharmacodynamics was stopped prior to the scheduled time if two consecutive MHb levels were at or below one percent. The time at which this occurred varied. Approximately 24 hr following dosing, an additional blood sample was taken.

Multiple Dose Pharmacodynamic Study (Phase VI): Within 48 hr of study initiation for each compound, dogs were weighed, and catheter placement sites clipped of hair. On dosing days, procedures such as study initiation, animal preparation, compound (WR242511 or PAHP) administration, and bleeding, were performed as previously described for pharmacodynamic studies except as follows. Each animal's weight was used to determine the target weight or volume of test compound placed in a gelatin capsule. Each capsule was prepared and placed into a scintillation vial (Kimble Glass, Inc., Vineland, NJ) labeled and color-coded for each dog. Food was removed for WR242511-dosed animals about 18 hr before dosing. For PAHP, food was removed approximately 4 hr prior to dosing. With both compounds, dogs were fed approximately 2 hr after dosing.

Blood samples were collected and analyzed as described in Study Memorandums dated June 1, 21, and 28, and July 23, 1994 of Appendix B. Additional blood volumes for hematocrit readings were taken periodically during scheduled blood draws to monitor blood loss. The initial and final dosings for each test compound were used also for limited pharmacokinetic experiments. A 20-ga, 1.25-in Abbocath® was placed in the cephalic vein of each dog for blood draws during the initial and final dosings. The catheter was attached to a three-way stopcock. For each compound, approximately 2-mL heparinized blood samples were collected in a-3 mL syringe. If blood collection from the catheter became difficult, a heparinized syringe with a 21-ga, 1-in needle was used to collect blood from another vein

(either cephalic and/or saphenous). Approximately 0.5 mL of the blood collected was used to analyze for Hb and MHb. The remainder of the blood sample was centrifuged for plasma collection. The plasma samples were labeled and stored at approximately -70 C until the end of the study, at which time plasma samples were shipped to WRAIR for compound analyses. Test compound analyses of plasma samples were to be performed by the sponsor and were not to be reported as a part of this study.

WR242511: For each animal, a loading dose of 2 mg of WR242511 crystals/kg BW was administered orally in gelatin capsules. Subsequent dosings were 1 mg/kg BW administered every 48 hr for two weeks. WR242511 dosing capsules were prepared within 24 hr of study initiation and stored as described in chemistry methods. All six animals were dosed in one day by staggering dosing (approximately 5 min) and bleeding times.

PAHP: PAHP was dissolved in PEG200 at a concentration sufficient to deliver the desired volume in one or two gelatin capsules. A target dose of 6.0 mg/kg BW was administered orally every 8 hr for up to nine days. Capsules were prepared periodically throughout the phase and stored as described in chemistry methods. On study day 8 (July 26, 1994) after the first dosing period, the amount of blood collected was reduced to approximately 1 mL because Hb levels and hematocrit readings indicated an anemic condition developing, presumably due to the multiple blood collections. More frequent blood collections were performed following the first and last dosing of PAHP as compared to the other dosings in order to obtain plasma samples for limited pharmacokinetic evaluation. All six animals were dosed in one day by staggering dosing (approximately 2 min) and bleeding times.

Efficacy Studies (Phases III, IV, V, and VIII): Procedures and methods discussed in Section 2.4 for Phase I were generally those used in the efficacy phases. Differences or additional procedures used in each phase are described. A heparin solution of 1,000 units/mL was diluted to approximately 2 units/mL for the saline flushes used in these phases.

Phase III - PAPP Efficacy: Each animal's weight was used to determine the PAPP dose on a mg/kg basis. An approximately 1-mL volume of PAPP or the vehicle (PEG200) was administered IV. The PAPP concentration varied depending upon the desired dosage and the weight of the dog dosed. Table 7 displays the PAPP doses and time following dosing to NaCN

infusion. The time following dosing prior to NaCN infusion varied depending upon the dose of PAPP administered and thereby the time to reach maximum MHb levels.

During Phase III, animal HFZADG was catheterized in the left jugular vein since palpation indicated an extremely small right jugular vein. In Phase III, the saphenous catheter was used to administer the pretreatment drug or vehicle (PEG200), followed by an approximately 3-mL saline flush, and also was used for the NaCN infusion. Depending upon the volume of NaCN estimated to be necessary to reach RA, a 30- or 60-mL syringe was used to infuse the NaCN solution. The hub of the syringe was connected to a 20 or 33 inch Medex-Inc-MX451-FL extension tube (Medex Inc., Hilliard, OH) prior to infusing NaCN. This type extension tube was used for the remainder of the efficacy experiments. For Phase III and the other efficacy phases, hydroxylamine was administered via the catheterized cephalic vein as opposed to the saphenous vein used in Phase I. There were no noticeable differences in the physiologic responses as a result of this change in vessel of administration. The Study Director believed this injection site of hydroxylamine to be more efficient because the injection was accomplished in closer proximity to the heart during a period of compromised circulation. Respiratory assistance and other symptomatic therapy were given as needed to support life following the 30-sec RA.

Phases IV and VIII - The dogs used in Phase IV were also used, for comparison purposes, in Phase VIII. Phase IV was completed approximately 1 month prior to the initiation of Phase VIII. Study Memorandums in Appendix B, dated April 1, 1994 for Phase IV and June 8, 14, and 17, 1994 for Phase VIII, provide additional performance details for these phases.

While it would have been ideal to randomize dosing of the short-acting PAPP and the long-acting WR242511 MHb-forming compounds over the second and third periods of Phase IV, the prolonged effect of the WR242511 precluded this approach. Therefore, the two MHb-forming compounds were examined in a systematic fashion. Generally, two animals per day were infused with a NaCN solution with a minimum one-week washout between each period except for between phases and for dogs given WR242511.

Pretreatment Administration: The vehicle, PEG200, was administered IV to each animal during Period 1 of Phase IV as described in Phase III Study Memorandum in Appendix B dated April 1, 1994. In Period 2 of Phase IV, PAPP at 0.2 mg/kg was administered IV to each animal. PAPP was dissolved in PEG200 at a concentration of 3 mg/mL with the exception of a 4.0 mg/mL preparation for one day's dosing. This was due to the heavier weight of the dogs. Once an animal was instrumented and stabilized, an approximately 1-mL volume of PAPP or the vehicle was administered via the catheterized cephalic vein, and this was followed by an approximately 3-mL saline flush. In Period 3 of Phase IV, WR242511 at 2.5 mg/kg was administered orally in gelatin capsules within 8 days prior to NaCN infusion. Each animal's body weight was used to determine the weight of WR242511 crystals to be placed into a gelatin capsule. In Phase VIII, doses and administration technique for PAHP were based on those used in Phase VII. The experimental parameters set for PAHP were those targeted for PAPP and WR242511 in Phase IV, using the oral route of administration. On each dosing day, at least one animal received the PEG200 vehicle. A stock dosing solution of PAHP in PEG200 was prepared at a concentration of approximately 120 mg/mL. This stock solution was used to dose animals in Phase VIII. Approximately 7 mg/kg BW of PAHP dissolved in PEG200 was administered orally in gelatin capsules. In general, one capsule, was used to administer the PAHP dose, but occasionally two capsules were required.

For Phase IV and VIII, the time delay between MHb-forming compound dosing and NaCN infusion varied depending upon the compound administered and the time at which the measured MHb level was within a range of 3 to 7 percent. PAPP and the vehicle were administered approximately 37 minutes prior to infusing NaCN. WR242511 was administered approximately 3 to 7 days prior to infusing NaCN. The MHb level in WR242511-dosed animals was monitored, beginning approximately 4 days after dosing, until the MHb was within the 3 to 7 percent range. In Phase VIII, when the MHb level measured approximately 3 percent, the animals were prepared for NaCN infusion. In general, the PAHP-dosed animal of Phase VIII was infused first, followed by the vehicle-dosed animal.

Day Prior to NaCN Infusion: Feed was removed approximately 18 hr prior to MHb-forming compound or NaCN dosing, and was returned approximately 2 hr after pretreatment dosing or after recovery from anesthesia.

Day of NaCN Infusion: Blood samples were collected periodically to measure Hb, MHb, and total blood CN^-. Blood samples for free CN^- measurement were not collected in Phase IV or the remainder of the study phases. In Phases IV and VIII, animals were prepared and instrumented, samples collected and analyzed, and NaCN infused as described for Phase I and in Study Memorandums in Appendix B dated April 1, 1994 (Phase IV), and June 8, 14, and 17, 1994 (Phase VIII).

Phase V: Phase V was designed to determine the efficacy of a MHb-forming compound in countering the lethal effects of NaCN at 2x AvTRA. PAPP pretreatment was chosen because of the ease of administration, the greater control over experimental variables, and the reduction in time to complete the experiment. The PAPP was dissolved in PEG200, and the techniques of administration were based on those used in Phases II, III, and IV. The PAPP solutions, prepared each day of experimentation, varied in concentration depending upon the weights of the dogs to be dosed. Approximately 0.3 mg PAPP/kg BW was administered IV to each animal.

Two animals per day were anesthetized, instrumented, pretreated, infused with NaCN, and treated as described for Phases I and III, and in Study Memorandums dated July 26, 1994 and August 12, 1994 in Appendix B. After stabilization, an approximately 1-mL volume of PAPP was administered IV over 1 min and followed by a 3-mL saline flush. The delay to CN infusion following PAPP dosing varied depending upon when the animal's MHb level approximated the target level of 6.5 percent.

The 2x AvTRA NaCN dose was determined using the collective data on untreated animals in Phase III experiments. A stock solution of NaCN in saline was prepared approximately 4 days prior to the start of this phase and analyzed for CN^- concentration. Individual aliquots of the NaCN stock solution were stored in a locked refrigerator. The NaCN stock solution concentration and each dog's weight were used to calculate the volume

that was programmed into the infusion pump to deliver 2x AvTRA. For NaCN infusion, a 30- or 60-mL syringe was used to draw a volume of 15-34 mL NaCN solution for infusion.

When the appropriate MHb level was reached, the 2x AvTRA NaCN dose was infused. If an animal stopped breathing prior to the complete infusion of the 2x AvTRA NaCN dose, the procedures used in previous phases (Phases III, IV, and VIII), i.e., use of the stop-watch and administration of hydroxylamine at 10 mg/kg 30 seconds after RA, were to be followed. In cases where RA did not occur, the animal would be allowed to recover without therapy. Blood samples were collected periodically to measure Hb and MHb, and total blood CN^-. The primary responses measured were TRA, MHb, and amount of NaCN administered.

2.5. Statistical Methods

Three primary endpoints were considered in statistical analyses to determine and compare the efficacies of PAPP, WR242511, and PAHP: TRA, the NaCN dose infused to cause RA, and percent MHb. In addition, six secondary endpoints were based on physiologic parameters and NaCN levels in the blood. Animal ID and treatment were included in analyses as explanatory variables. Table 11 provides a description of each primary and secondary endpoint, and the explanatory variables.

Three types of statistical models were fitted to the data:

1) Analysis of variance models,
2) Regression models, and
3) Nonlinear pharmacodynamic compartment models.

All of the statistical analyses were implemented using the Statistical Analysis System (SAS; Version 6.08, Cary, NC). Tables 12, 13, and 14 present the statistical models fitted to the data in each of the study phases.

Feasibility Study (Phase I): Two-way analyses of variance (Table 12) were conducted to assess the animal and study week effects on TRA and baseline Hb. Mixed analysis of

TABLE 11. DESCRIPTION OF ENDPOINTS ANALYZED

Parameter	Endpoint	Description
Efficacy	Time to Respiratory Arrest[a] (min)	Time until breathing stops.[b]
	Infused NaCN (mg/kg)	Total NaCN dose infused to cause cessation of respiration.
	Percent MHb (%)	Percent MHb immediately prior to NaCN infusion.
Heart Rate	Time to Peak (min)[a]	Time to peak heart rate based on moving averages of the PO-NE-MAH heart rate data collected between the start of NaCN infusion and respiratory arrest.
	Peak Shift from Baseline[c] (beats per minute)	Baseline heart rate subtracted from the peak heart rate observed between start of NaCN infusion and respiratory arrest.
Respiratory Rate	Time to Peak[a] (min)	Time to peak respiratory rate based on moving average PO-NE-MAH data collected between start of NaCN infusion and RA.
	Peak Shift from Baseline[c] (breaths per minute)	Baseline respiratory rate subtracted from the peak respiratory rate observed between start of NaCN infusion and RA.
Total Blood CN^-	Average Level After Respiratory Arrest (μg/mL)	Average of Total Blood CN^- during the first hour following respiratory arrest.
	Uptake (μg/mL/min)	Rate of increase of Total Blood CN^- during NaCN infusion, estimated by linear regression of Total Blood CN^- over time.
Free CN^-	Time to Peak[a] (min)	Time to peak Free CN^- measurement in blood samples collected during NaCN infusion.
	Peak (μg/mL)	Peak Free CN^- measurement.
Explanatory Variables	Treatment	Targeted dose of MHb-inducing compound (mg/kg).
	Animal ID	Unique laboratory animal identifier

[a] Time measured from beginning of NaCN infusion.
[b] Time to respiratory arrest was defined as the elapsed time between the beginning of NaCN infusion and the stopping of infusion when no functional breaths has been observed for 10 sec.
[c] Baseline was calculated as the mean of the PO-NE-MAH readings in the two-min period immediately prior to NaCN infusion.

variance models (Table 12) were fitted to the baseline data for heart rate and respiratory rate to assess the extent of any trends over dosing weeks. These models estimated the overall trends

in the measured parameters over weeks of dosing, but allowed for the random variation among animals and across dose weeks within animals.

TABLE 12. ANALYSIS OF VARIANCE MODELS FITTED TO DATA

Phase	Endpoint	Effects in Model	Parameters	Program
I	TRA Baseline Hb	$\mu + \beta + \gamma + \epsilon$	μ = average β = random effect of animal γ = week effect ϵ = error	Proc GLM
II PAPP	Empiric Peak % MHb Modelled Peak % MHb Empiric Time to Peak Modelled Time to Peak	$\mu + \beta + \prec + \tau + \epsilon$	μ = average β = random effect of animal $\prec$ = effect of PAPP dose τ = effect of dosing sequence ϵ = error	Proc GLM
II WR242511	ln(Empiric Peak % MHb) ln(Modelled Peak % MHb) Empiric Time to Peak Modelled Time to Peak	$\mu + \beta + \prec + \tau + \epsilon$	μ = average β = random effect of animal $\prec$ = effect of WR242511 dose τ = effect of dosing sequence ϵ = error	Proc GLM
III	ln(TRA) ln(Infused CN dose) Percent MHb ln(Time to Peak HR) ln(Time to Peak RR) Peak shift from baseline HR Peak shift from baseline RR ln(Average Total Blood CN^- after Respiratory Arrest) Uptake of Total Blood CN^- ln(Time to Peak Free Plasma CN^-) Peak Free Plasma CN^-	$\mu + \beta + \alpha + \tau + \epsilon$	μ = average β = random effect of animal α = effect of PAPP dose τ = effect of dosing sequence ϵ = error	Proc GLM
IV VIII	ln(TRA) ln(Infused CN dose) Percent MHb	$\mu + \beta + \alpha + \epsilon$	μ = average β = random effect of animal α = treatment effect ϵ = error	Proc GLM
VII	Empiric Peak % MHb Modelled Peak % MHb Empiric Time to Peak Modelled Time to Peak	$\mu + \beta + \alpha + \epsilon$	μ = average β = random effect of animal α = effect of PAHP dose ϵ = error	Paired t-tests

TABLE 13. REGRESSION TYPE MODELS FITTED TO DATA

Phase	Endpoint	Effects in Model	Parameters	Program
II	Modelled peak percent MHb Modelled time to peak MHb	$\beta_o + \beta_1$Ln(PAPP Dose)	β_0 = intercept β_1 = slope ln = natural log	Proc Reg
III	ln(TRA) ln(Infused CN dose)	$\beta_o + (\beta_1 + \beta_i)$*(%MHb)	β_0 = intercept β_1 = average slope β_i = random slope adjustment for ith animal	Proc Mixed
IV VIII	TRA Infused CN dose	$\beta_{oi} + \beta_{1i}$ *(%MHb)	β_{0i} = intercept for ith treatment β_{1i} = slope for ith treatment i = PAPP, WR24211, or PAHP	Proc Mixed using repeated covariance structure to model the intra-animal correlation

TABLE 14. NONLINEAR MODELS FITTED TO DATA

Phase	Endpoint	Effects in Model	Parameters	Program
II PAPP	% MHb over Time	B (e-ket + e-kat)	B = model parameter ka = absorption rate constant ke = elimination rate constant t = time	Proc Nlin
II WR242511	% MHb over Time	(A+Bt) e-kt	A = model parameter B = model parameter k = model parameter t = time	Proc Nlin
VII PAHP	% MHb over Time	(A+Bt)e-kt	A = model parameter B = model parameter k = model parameter t = time	Proc Nlin
VI Multiple Dosing	% MHb over Time	C e-(a+bt)	a = model parameter b = model parameter C= model parameter t = time	Proc Nlin

Single Dose Pharmacodynamic Studies (Phases II and VII): Time-course plots of PAPP, WR242511, and PAHP data for each experiment indicated an increasing MHb concentration, followed by a slower decrease in concentration after reaching a peak. For each

experiment, a one-compartment pharmacodynamic model (Table 14) was fitted to the percent MHb data. In most cases, the fitted model explained more than 95 percent of the variability in the MHb data.

For each animal, pretreatment compound, and dose, the fitted model was used to estimate peak percent MHb and time to peak. In addition, the peak percent MHb and time to peak were estimated from the empiric time course data. For WR242511 and PAPP, the empiric and modelled peak percent MHb and time to peak values were statistically analyzed to determine if there were any effects due to dose or sequence of testing, and to assess the animal-to-animal variability. Analysis of variance models fitted to the WR242511 and PAPP data are displayed in Table 12. For WR242511, the variability in peak percent MHb appeared to increase with increasing WR242511 dose, and therefore the analysis of variance was conducted on natural logarithm (ln) transformed values. For each parameter, hypothesis tests were performed to determine if the animal-to-animal or dosing sequence variability were statistically significant. Since there were only 2 dose levels for PAHP, the empiric and modelled peak means were compared using paired t-tests (ProcMeans).

For PAPP, a simple linear regression model (Table 13) was fitted to the modelled peak percent MHb data using ln PAPP dose as the independent variable. The fitted model was used to estimate the PAPP doses that would produce 2.5, 5, and 10 percent MHb. A similar regression was fitted to the modelled time to peak MHb data. The fitted model was used to predict times to peak MHb for the PAPP doses estimated to produce 2.5, 5, and 10 percent MHb. Linear regression models were not fitted to WR242511 or PAHP data.

Multiple Dose Pharmacodynamic Study (Phase VI): For each animal, an exponential model (Table 14) was used to smooth the percent MHb data over time. Model parameters were used to estimate the SS level of percent MHb and time until the percent MHb reached 95 percent of the SS level (t_{95}) for each animal. The standard error for t_{95} was calculated using Taylor series approximation.[14]

For WR242511, the exponential model was fitted to the percent MHb data over time for each animal. The SS level estimated by the model represents the average percent MHb

level after reaching SS. The estimate of parameter C represents the average percent MHb at SS (SS average).

For PAHP, MHb levels, in general, exhibited the typical pharmacological profile of a one compartment open model with first order absorption. Therefore, MHb levels were quite variable in each dose period: initially increasing to a peak followed by a decrease. To calculate the minimum level of protection for each animal, the percent MHb at SS was estimated as follows. The minimum percent MHb level following each dose, called the trough in this report, was calculated and modelled over time. An exponential model (Table 14) was fit to the trough data. The estimate of parameter C for the models fitted to the PAHP data represents the trough percent MHb at SS (SS trough).

Because the variability in the modelled SS and t_{95} levels was large for some animals, weighted mean SS and t_{95} levels were calculated for each test compound. The weighted mean and standard error were calculated where x_i is the modelled SS or t_{95} for the *ith* animal and

$$\bar{x}_w = \frac{\sum_{i=1}^{n} w_i x_i}{\sum_{i=1}^{n} w_i}$$

$$SE_w = \sqrt{\frac{\sum_{i=1}^{n} w_i (x_i - \bar{x}_w)^2}{(n-1)\sum_{i=1}^{n} w_i}}$$

w_i is one over the variance of either the modelled SS or t_{95} for the *ith* animal. These weights place less emphasis on observations with high variability.

Efficacy Studies (Phases III, IV, V, and VIII): Phase III - Analyses of variance (Table 12) were carried out to detect significant effects for PAPP dose, dosing sequence, and animal-to-animal variability. In addition, Dunnett's test[15] was used for two-sided pairwise comparisons between treatment and control means. Dunnett's test adjusts for multiple

comparisons, so that the experimentwise error rate for the tests was controlled at the 0.05 level of significance.

A linear regression model (Table 13) in percent MHb with random slopes for each animal was fitted to the TRA and infused NaCN dose data. This model treated the data as 8 sets of 4 repeated measures rather than 32 independent observations. The intercept and mean slope were used to describe the relationship between the response and percent MHb for an average animal.

Phases IV and VIII - Analysis of variance models (Table 12) were fitted to the combined data from Phase IV and Phase VIII experiments to determine if there were effects due to treatment, to compare the effects of PAPP, WR242511, and PAHP, and to assess the animal-to-animal variability. Tukey's test[15] was used for pairwise comparisons between treatment and control means, between the two sets of control means, and between the PAPP, WR242511, and PAHP treatment means.

Linear regression models (Table 13) that accommodated the correlation between multiple observations on the same animal were fitted to the PAPP, WR242511, and PAHP data. The intra-animal correlations were modeled with a repeated covariance structure. T-tests were used to make pairwise comparisons between estimated intercepts and slopes for PAPP, WR242511, and PAHP.

Phase V - Tables of descriptive statistics and plots were prepared for the primary and secondary endpoints.

3.0 RESULTS

Tables and figures of results are presented in a separate appendix for each phase. Summary tables and figures for a phase or combined phases appear in the text below.

Feasibility Study (Phase I): Data tables, descriptive statistics, and figures for Phase I experiments are included in Appendix D, Phase I - Data and Statistical Analyses. Table D-1 of Appendix D presents the TRA and the NaCN infusion dose for each animal on each dosing day. Descriptive statistics for TRA are displayed for each animal in Table D-2 and for each week of dosing in Table D-3. Figures D-1 through D-4 display the MHb levels plotted against

time for each animal.

The secondary responses are plotted against time for each animal in Figures D-5 through D-24. Time course plots of the heart rate and respiratory rate data did not reveal the presence of any linear increases or decreases in baseline levels prior to dosing, although the average baseline heart and respiratory rates varied considerably across experiments within an animal. Table 15, below, summarizes the results of fitting analysis of variance models to the TRA, heart rate, respiratory rate, and hemoglobin data.

Weekly means for TRA ranged between 2.27 min and 2.45 min. While the effect of animal variability on TRA was statistically significant (p-value = 0.04), the effect of week of dosing was not significant. Figure 2 displays TRA data for each animal for each week of dosing. These data indicated that TRA was relatively constant for this repeated dosing protocol, except for animal HFXAYK although the inter-animal variability was significant.

TABLE 15. PHASE I-RESULTS OF STATISTICAL ANALYSIS

	Animal Variability		Mean (Week)				Week of Dosing Effect	
	σ_A	p-value	1	2	3	4	F-statistic	p-value
TRA	0.27	0.04	2.45	2.28	2.45	2.27	0.47	0.71
Heart Rate	15.95	0.38	115.00	96.00	106.00	109.00	0.67	0.59
Respiratory Rate	1.41	0.88	20.00	17.00	15.00	20.00	0.50	0.69
Hemoglobin	0.61	0.43	12.99	10.21	9.29	10.51	12.68	<0.01

As shown in Table 15, the overall week of dosing effect was not statistically significant for either heart rate or respiratory rate. Thus, the experiment did not yield strong evidence of a residual effect on either baseline heart rate or baseline respiratory rate over the repeated dosings. The week of dosing effect was statistically significant (p-value < 0.01) for the baseline Hb levels. This can be seen in Figures D-21 through D-24 in Appendix D, where the baseline Hb levels are higher in the first experiment when compared with the three later experiments for each animal. This was probably a result of the frequent blood draws from each animal.

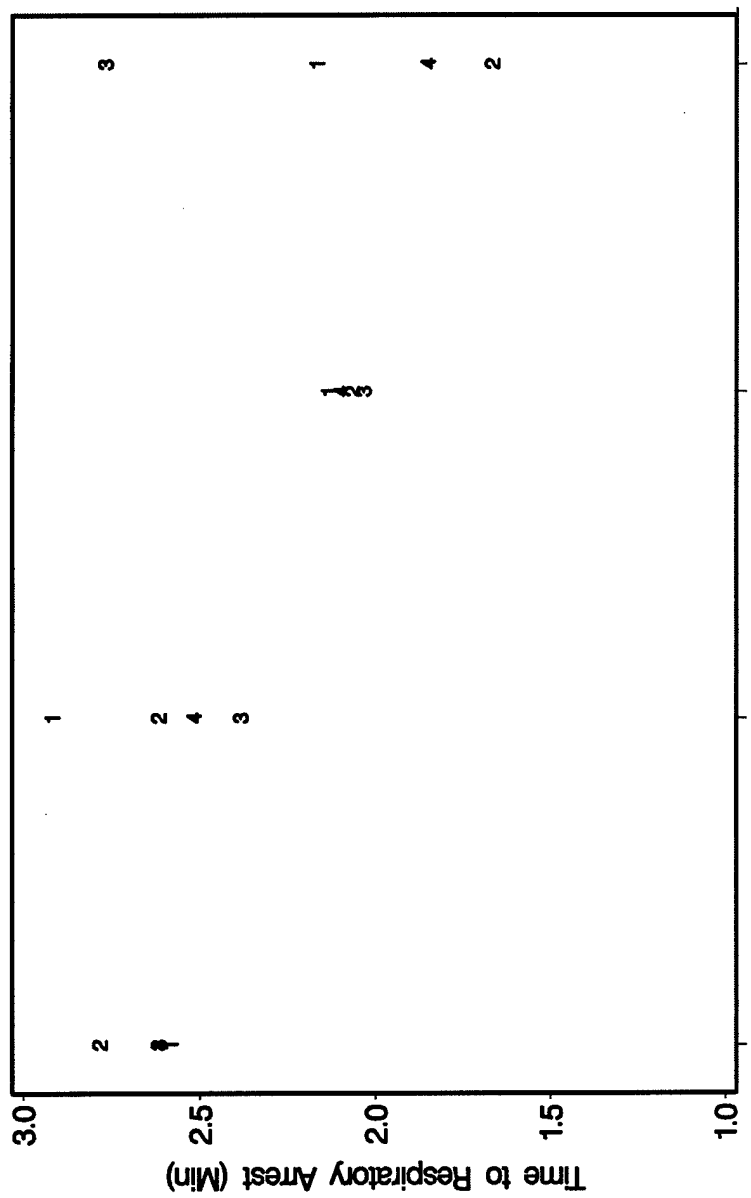

FIGURE 2. PHASE I-TIME TO RESPIRATORY ARREST FOR EACH ANIMAL, WITH WEEK OF EXPERIMENT INDICATED BY PLOTTING SYMBOL

Single Dose Pharmacodynamic Studies (Phases II and VII): Tables of data, descriptive statistics, and figures for Phase II experiments (PAPP and WR242511) are included in Appendix E, Phase II-Data and Statistical Analyses. PAPP experimental results are presented first in this section and in the appendix, followed by experimental results for WR242511. Similar tables and figures for Phase VII experiments are presented in Appendix F, Phase VII-Data and Statistical Analyses. Data and results for the Phase VII pilot experiment are given also in Appendix F.

Phase II - PAPP: Table E-1 presents the data for PAPP experiments. Figures E-1 through E-4 display time course plots of the modelled PAPP dose-MHb curves overlaid on the observed MHb percentages for each animal. Table E-2 presents the modelled and empiric values for peak percent MHb and time to peak, as well as the modelled absorption and elimination half-lives. Modelled peak percent MHb and time to peak were plotted against PAPP dose in Figures E-5 and E-6, respectively. Descriptive statistics for the empiric and modelled peak percent MHb and time to peak at each of the four dose levels of PAPP are displayed in Table E-3.

Table 16 summarizes the results of statistical analyses and hypothesis testing for the effects of PAPP dose and dosing sequence, and the animal-to-animal variability. Columns 3-8 present the results for dose effect on empiric and modelled peak percent MHb and time to peak. The average value of the response parameter is given for each dose level in columns 3-6. For each parameter, a hypothesis test was performed to determine if the dose effect was statistically significant. The values of the F statistics and their observed significance levels are given in the next two columns of the table. The effect of the PAPP dose was statistically significant for all parameters.

The animal-to-animal variability is summarized in columns 9-11 of Table 16. The estimated animal-to-animal variability is reported in column 9. The ratio of animal-to-animal variance to uncontrolled error variance is given in the next column. For each parameter, a hypothesis test was performed to determine if the animal-to-animal variability was statistically significant. The significance levels of these tests are reported in column 11. The

TABLE 16. PHASE II-SUMMARY OF STATISTICAL ANALYSIS OF PAPP DOSE, ANIMAL TO ANIMAL VARIABILITY, AND DOSE SEQUENCE FOR EMPIRIC AND MODELLED PEAK PERCENT METHEMOGLOBIN AND TIME TO PEAK

		PAPP Dose Effect						Animal Variability [a]			Dose Sequence Effect	
		PAPP Dose (mg/kg)										
Method	Parameter	0.10	0.13	0.22	0.37	F-Stat	p-value	σ^2_A	σ^2_A/σ^2_E	p-value	F-Stat	p-value
Empiric	Peak Percent Methemoglobin (%)	2.0	3.6	6.0	9.9	126.7	0.000	2.2	6.1	0.001	3.2	0.105
	Time to Peak (min)	22.5	27.5	45.0	60.0	20.1	0.002	20.8	0.4	0.164	1.6	0.291
Modelled	Peak Percent Methemoglobin (%)	2.1	3.7	6.2	10.4	109.9	0.000	2.4	5.1	0.001	3.1	0.113
	Time to Peak (min)	26.7	32.6	41.4	58.2	38.6	0.000	6.7	0.4	0.168	1.6	0.288

[a] σ^2_A = Estimate of the animal-to-animal variance component.

σ^2_A/σ^2_E = Ratio of the variance components estimated for animals to the variance component estimated for uncontrolled error.

p-value = Observed significance level for the animal-to-animal variance component.

animal-to-animal variability was statistically significant for the empiric and modelled peak percent MHb parameters, but was not significant for the time to peak MHb parameters.

For each parameter, a hypothesis test was performed to determine if the dosing sequence had a statistically significant effect. The values of the F statistics and their observed significance levels are presented in the last two columns of Table 16. The dosing sequence did not have a statistically significant effect for any of the parameters.

Figures E-7 and E-8 display the modelled peak percent MHb and time to peak MHb values plotted against the ln PAPP dose together with the fitted regression lines. The intercept and slope were estimated to be 16.4 and 6.5, respectively, for the linear regression model fitted to the peak percent MHb data. Similarly, the intercept and slope were estimated to be 80.1 and 24.3, respectively, for the modelled time to peak data. Table 17 presents the PAPP doses predicted to produce peak percent MHb levels of 2.5, 5, and 10 and the predicted time to peak for each PAPP dose. For this analysis, the PAPP dose was based on the chemical analysis of the PAPP concentration for each experiment.

WR242511: Table E-4 presents the data for WR242511 experiments. Figures E-9 through E-12 display time course plots of the modelled WR242511 dose curves overlaid on the observed percent MHb for each animal. Unusually high percent MHb values were observed for animal HFXBGI at the 2.5 mg/kg dose, animal HFYBCP at the 7.4 mg/kg dose, animal HFYBEI at the 1.3 mg/kg dose, and animal HFZBFI at the 5.0 mg/kg dose. All of these unusually high observed values occurred four days after dosing in the first week of the experiment (Friday, October 29, 1993). On October 29, 1993 the four MHb samples apparently were run in the human MHb mode of the Hemoximeter® and not the dog mode. These values were included when fitting the models to smooth the percent MHb data, but were not included when calculating the empiric values described below.

The modelled and empiric values of peak percent MHb and time to peak are presented in Table E-5. The ln-transformed empiric and modelled peak percent MHb are plotted against WR242511 dose in Figures E-13 and E-14. Peak percent MHb levels were greater than five percent for each animal at the 2.5 mg/kg WR242511 dose, and peak levels were reached approximately 3 days after the dose was administered. The MHb levels returned to

approximately five percent at 7, 5, 6 and 6 days in animals HFXBGI, HFYBCP, HFYBEI, and HFZBFI, respectively, after the 2.5 mg/kg dose. Time to peak data did not exhibit a trend

TABLE 17. PHASE II-ESTIMATED PAPP DOSE PRODUCING 2.5, 5, AND 10 PERCENT METHEMOGLOBIN

Percent MHb(%)	PAPP Dose[a] (mg/kg)	Time to Peak[b] (min)
2.5	0.12	28
5.0	0.17	38
10.0	0.37	56

[a] PAPP dose predicted to produce specified peak percent MHb.
[b] Predicted time to peak for specified peak percent MHb.

with WR242511 doses. Descriptive statistics for the empiric and modelled peak percent MHb and time to peak at each of the four doses of WR242511 are displayed in Table E-6.

Table 18 summarizes the results of the statistical analyses and hypothesis testing for WR242511. The structure of the table is identical to that described for Table 16. Natural log-transformed values for the empiric and modelled peak percent MHb were used in the analysis. The effect of the WR242511 dose was statistically significant at the 5 percent level for both the empiric ($p = 0.002$) and modelled ($p = 0.002$) peak percent MHb. In both empiric and modelled cases, the average of the ln-transformed peak percent MHb values at the 1.3 mg/kg dose was significantly less than that observed at the other dose levels, while no differences were observed among the higher dose levels. The dose effect was not statistically significant for the empiric or modelled time to peak. Neither the animal-to-animal variability nor the effect of dosing sequence were statistically significant at the 5 percent level for any of the parameters.

Phase VII - MHb and Hb data are provided in Table F-1. Figures F-1 through F-6 display time course plots of the modelled percent MHb curves overlaid on the observed percent MHb levels for each animal.

TABLE 18. PHASE II-SUMMARY OF STATISTICAL ANALYSIS OF THE EFFECTS OF WR242511 DOSE, ANIMAL-TO-ANIMAL VARIABILITY, AND DOSE SEQUENCE ON EMPIRIC AND MODELLED PEAK PERCENT METHEMOGLOBIN AND TIME TO PEAK

		WR242511 Dose Effect						Animal Variability[a]			Dose Sequence Effect	
		WR242511 Dose (mg/kg)										
Method	Parameter	1.3	2.5	5.0	7.4	F-Stat	p-value	σ^2_A	σ^2_A/σ^2_E	p-value	F-Stat	p-value
Empiric	Peak Percent[b] Methemoglobin (%)	3.5[c]	7.9	14.2	12.3	18.8	0.002	0.04	0.44	0.135	2.1	0.207
	Time to Peak (days)	2.3	2.8	3.0	3.0	2.0	0.215	0.00	0.00	0.602	2.0	0.217
Modelled	Peak Percent[b] Methemoglobin (%)	3.3[c]	7.7	14.1	12.2	19.3	0.002	0.04	0.45	0.131	1.9	0.228
	Time to Peak (days)	2.8	2.9	3.1	3.1	3.1	0.111	0.03	0.75	0.071	2.1	0.200

[a] σ^2_A = Estimate of the animal-to-animal variance component.

σ^2_A/σ^2_E = Ratio of the variance components estimated for animals to the variance component estimated for uncontrolled error.

p-value = Observed significance level for the animal-to-animal variance component.

[b] Statistical analysis was conducted on ln-transformed values for this endpoint. Estimated geometric means are reported for each WR242511 dose. The animal-to-animal variability is estimated for the ln-transformed data.

[c] Average of ln-transformed values for the 1.3 mg/kg dose was less than those observed for the 2.5, 5.0, and 7.4 mg/kg dose groups at the 0.05 significance level. No significant differences were observed among the higher dose levels.

TABLE 19. PHASE VII-STATISTICAL COMPARISON OF EMPIRIC AND MODELLED PEAK PERCENT METHEMOGLOBIN AND TIME TO PEAK FOR 2.0 AND 5.0 mg PAHP/kg EXPERIMENTS

Method	Endpoint	Difference in Means	Standard Error	p-value
Empiric	Peak Percent Methemoglobin (%)	4.2	1.5	0.037
	Time to Peak Methemoglobin (hours)	0.1	0.3	0.785
Modelled	Peak Percent Methemoglobin (%)	4.0	1.4	0.039
	Time to Peak Methemoglobin (hours)	0.2	0.2	0.283

Modelled and empiric estimates of peak percent MHb and time to peak are presented in Table F-2. Table F-3 provides descriptive statistics for the empiric and modelled peak percent MHb and time to peak at each PAHP dose. The mean and standard deviation of modelled peak percent MHb at 2 mg PAHP/kg were 3.28 percent and 1.26, respectively. The mean and standard deviation of modelled peak percent MHb at 5 mg PAHP/kg were 7.29 percent and 3.32, respectively, although modelled peak percent MHb at 5 mg PAHP/kg in individual animals ranged from 3.1 to 11.4 percent.

Results of the paired t-test hypothesis testing are reported in Table 19. Although the difference was small for some animals, the mean peak percent MHb at 5.0 mg PAHP/kg was statistically greater (5 percent significance level) than that at 2.0 mg PAHP/kg for both the empiric and modelled peaks. There were no significant differences in the empiric or modelled times to peak.

Multiple Dose Pharmacodynamic Study (Phase VI): Data tables, descriptive statistics, and figures for Phase VI experiments (WR242511 and PAHP) are presented in Appendix G, Phase VI-Data and Statistical Analyses. WR242511 experimental results are presented in this section and in the appendix first, followed by experimental results for PAHP.

WR242511: The percent MHb time course data for WR242511 experiments are displayed in Table G-1. Figures G-1 through G-6 display the modelled curves overlaid on the observed percent MHb levels over time for the 6 animals treated with WR242511. The horizontal line on each plot indicates the modelled SS average. Table 20 presents the SS

average and t_{95} for animals treated with WR242511. The weighted mean and standard error over these six animals are shown in the last line of the table.

TABLE 20. PHASE VI-STEADY STATE AVERAGE PERCENT METHEMOGLOBIN AND TIME TO 95 PERCENT OF STEADY STATE LEVEL (t_{95}) FOR ANIMALS TREATED WITH WR242511[a]

	Steady State Average Percent MHb (%)		t_{95} (days)	
Animal ID	Estimate	SE	Estimate	SE
HFXAYH	10.89	0.14	7.34	0.48
HFXAYK	9.46	0.25	11.34	1.29
HFXBAD	10.79	0.14	7.21	0.60
HFXBGH	15.93	0.16	7.99	0.40
HFZADI	13.64	0.12	7.54	0.42
HFZADP	8.60	0.18	16.25	0.97
	Mean	SE	Mean	SE
Weighted Mean Over All Animals	12.04	1.04	8.14	0.90

[a] Dogs were given 2 mg WR242511 crystals/kg BW loading dose per os followed by 1 mg/kg per os every 48 hr for 2 weeks.

PAHP: The percent MHb time course data for PAHP experiments are provided in Table G-2. Figures G-7 through G-12 display the modelled curves overlaid on the measured percent MHb levels over time for the six animals treated with PAHP. The horizontal line on each plot denotes the estimated SS trough. Table 21 presents the estimated SS trough and t_{95} for animals treated with PAHP. The weighted mean and standard error over these six animals are reported in the last row of the table. An empiric estimate of the SS trough percent MHb was calculated as the median of the observed troughs, excluding the first four and last two dose periods. The first four dose periods were excluded because the MHb level was clearly rising during those periods. The last two dose periods were excluded because some animals were not dosed in the last one or two days of the scheduled 9-day experiments. The median trough percent MHb for each animal is presented in Table 21. The unweighted mean and standard error of the median trough percent MHb over these animals are displayed in the last row of the table. Experiments were terminated early in some dogs when signs of anemia were observed.

For animals receiving PAHP, the baseline hematocrit sample indicated the animals were within the expected normal range of approximately 37-55 percent.[16]

TABLE 21. PHASE VI-MEDIAN AND STEADY STATE TROUGH PERCENT METHEMOGLOBIN AND TIME TO 95 PERCENT OF STEADY STATE LEVEL (t_{95}) FOR ANIMALS TREATED WITH PAHP[a]

	Steady State Trough Percent MHb (%)				t_{95} (days)	
		Steady State				
Animal ID	Median	Estimate	SE		Estimate	SE
HFXAGU	6.50	6.65	0.43		2.31	0.87
HFXBGI	16.55	15.58	1.20		2.05	1.01
HFYBCP	13.30	23.48	11.86		19.93	19.13
HFYBEI	7.90	8.98	1.11		5.80	2.95
HFZAYM	12.70	12.68	0.42		2.28	0.48
HFZBFI	5.80	6.82	0.60		5.01	1.95
	Average Median	Median SE	Weighted Mean	SE	Weighted Mean	SE
	10.46	1.77	9.42	1.39	2.42	0.34

[a] PAHP was dissolved in PEG200 and given at 6 mg/kg BW in gelatin capsules every 8 hr for up to 9 days.

Efficacy Studies (Phases III, IV, V, and VIII): Data tables, descriptive statistics, and figures for Phase III experiments are presented in Appendix H, Phase III-Data and Statistical Analyses. Similar tables and figures for each individual animal for Phase IV experiments are presented in Appendix I, Phase IV-Data and Statistical Analyses. Tables and figures for Phase VIII experiments are presented in Appendix J, Phase VIII-Data and Statistical Analyses, and for Phase V experiments in Appendix K, Phase V-Data and Statistical Analyses. Data tables, descriptive statistics, and figures used in the text are not repeated in the appendices. Results of the efficacy studies conducted for Phases III, IV, VIII, and V are summarized in the appropriate section below. Phases IV and VIII data have been combined for the summary section.

Phase III - Three measures of efficacy were employed to examine the effect of PAPP dose: TRA, infused NaCN dose, and percent MHb. Table H-1 presents the TRA, NaCN infusion dose, and percent MHb data. Descriptive statistics for these parameters are provided in Table H-2.

Table 22 summarizes the ANOVA results for the efficacy parameters. Analysis of variance models were fitted to the TRA, infused NaCN dose, and percent MHb data with PAPP dose and dose sequence as explanatory variables and a random effect for animal-to-animal variability.

TABLE 22. PHASE III-SUMMARY OF THE STATISTICAL ANALYSES OF THE EFFECTS OF PAPP DOSE, ANIMAL-TO-ANIMAL VARIABILITY, AND DOSING SEQUENCE ON INFUSED NaCN DOSE, TIME TO RESPIRATORY ARREST AND PERCENT MHb

Endpoint	PAPP Dose Effect					Animal Variability[a]		Dosing Sequence[b]
	PAPP Dose (mg/kg)							
	0	0.12	0.17	0.37	p-value	σ_A	p-value	p-value
TRA (min)[c]	2.36	3.64[d]	4.32[d]	9.30[d]	<0.001	0.16	<0.001	0.110
Infused NaCN (mg/kg)[c]	1.52	2.30[d]	2.74[d]	6.27[d]	<0.001	0.06	0.265	0.286
Percent MHb (%)	0.00	2.90[e]	4.16[e]	11.33[e]	<0.001	0.55	0.010	0.205

[a] σ_A = Estimate of the animal-to-animal standard deviation, and p-value = Observed significance level for the animal-to-animal variance component.
[b] p-value for the significance of the dosing sequence.
[c] Statistical analysis was conducted on ln-transformed values for this endpoint. Estimated geometric means are reported for each PAPP dose. The animal-to-animal ln-standard deviation estimate is reported.
[d] Average of ln-transformed values was significantly greater than that observed for the 0 mg/kg control at the 0.05 significance level.
[e] Average value was significantly greater than that observed for the 0 mg/kg control at the 0.05 significance level.

Because the variability in TRA and infused NaCN dose appeared to increase with PAPP dose, the analysis of variance was conducted on ln-transformed values for these endpoints. Ln-transformed TRA and infused NaCN dose are plotted as a function of PAPP dose in Figures H-1 and H-2. Percent MHb was plotted as a function of PAPP dose in Figure H-3.

The results of statistical analyses on the effect of PAPP dose are presented in columns 2-6 of Table 22. Mean percent MHb is presented for each PAPP dose, and geometric means

are displayed for TRA and infused NaCN dose. The effect of PAPP dose was statistically significant for all three endpoints ($p < 0.001$ in each case) based on the F-test for dose effect. As indicated in the table, mean levels of TRA, infused NaCN dose, and percent MHb at each PAPP dose were significantly greater than those observed for the control 0 mg/kg dose at the 0.05 significance level based on Dunnett's Multiple Comparisons Test.

The estimated animal-to-animal standard deviation and corresponding p-value are displayed in columns 7 and 8, respectively. Animal variability was significant for TRA ($p < 0.001$) and percent MHb ($p = 0.010$). Animal variability was not significant for infused NaCN dose. Dosing sequence was not significant at the 0.05 level for any of the efficacy parameters as shown by the p-values in column 9.

To explore the relationship between TRA and percent MHb, a linear regression model with random slopes for each animal was fitted to the data. This model treats the data as 8 sets of 4 repeated measures rather than 32 independent observations. The following equation describes the modelled relationship between TRA and percent MHb for an average animal:

$$Ln(\textit{Time to Respiratory Arrest}) = 0.92 + 0.12\ (\textit{Percent MHb})$$

The model was fitted to ln-transformed values for TRA because increasing variability in TRA occurred with increasing percent MHb. The mean line is shown in Figure 3, with individual observed values of ln-transformed TRA and percent MHb.

A similar model was used to examine the relationship between infused NaCN dose to attain RA and percent MHb. Figure 4 displays the ln-transformed infused NaCN dose plotted against percent MHb with individual observed values overlaid on the mean regression line. The estimated intercept and slope for an average animal are 0.46 and 0.12, respectively.

Physiologic Parameters: The effect of PAPP dose on heart and respiratory rates was evaluated by examining the time to peak and the peak shift from baseline heart and respiratory rates. Descriptive statistics for these endpoints are presented in Table H-3. Moving average heart rates are plotted against time for each animal, labeled for PAPP dose, in Figures H-4

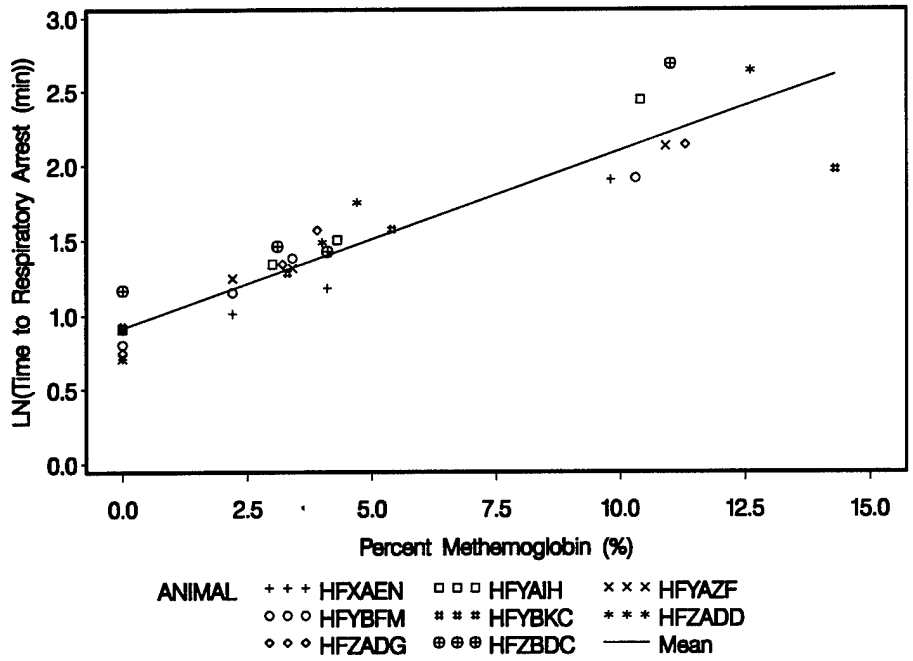

FIGURE 3. PHASE III-Ln (TIME TO RESPIRATORY ARREST) PLOTTED AGAINST PERCENT MHb. OBSERVED VALUES OVERLAID ON MEAN REGRESSION LINE.

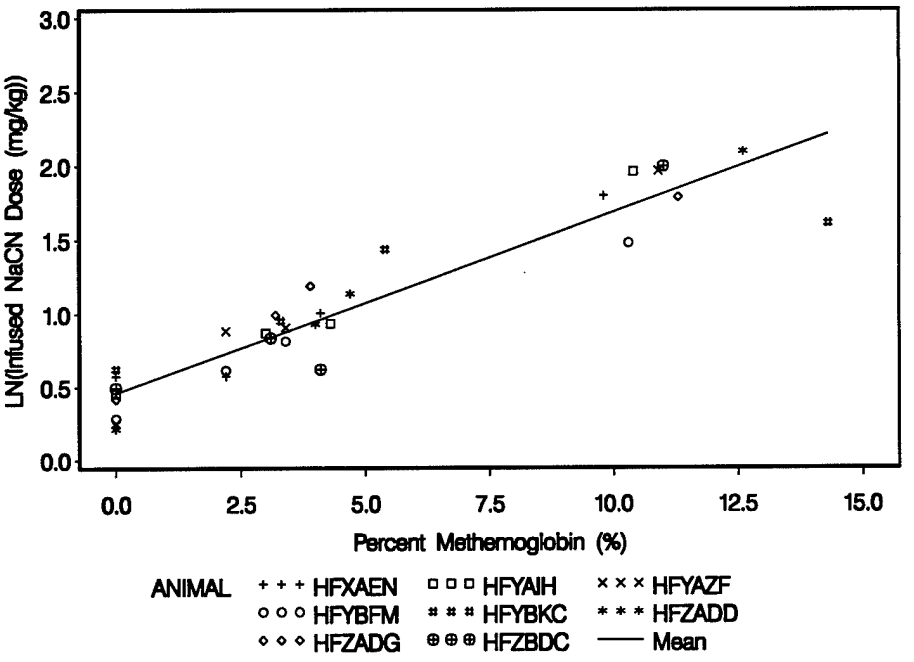

FIGURE 4. PHASE III-Ln (INFUSED NaCN DOSE) PLOTTED AGAINST PERCENT MHb. OBSERVED VALUES OVERLAID ON MEAN REGRESSION LINE.

through H-11, and moving average respiratory rates are plotted against time in Figures H-12 through H-19.

Analysis of variance models were fitted to times to peak and peak shifts from baseline heart and respiratory rates with PAPP dose and dose sequence as explanatory variables and a random effect for animal-to-animal variability. Results of the analyses of variance are summarized in Table 23. Because the variability in times to peak for heart and respiratory rates appeared to increase with PAPP dose, ln-transformed values were analyzed for these endpoints. Geometric means at each PAPP dose and the estimated animal-to-animal standard deviation of the ln-transformed values are shown in Table 23 for the times to peak data. Ln-transformed times to peak and peak shifts from baselines are plotted against PAPP dose in Figures H-20 and H-21 for heart rate and Figures H-22 and H-23 for respiratory rate. The average ln-transformed time to peak heart rate was statistically greater at the 0.17 and 0.37 mg/kg PAPP doses when compared to the 0 mg/kg control dose based on Dunnett's Multiple Comparisons Test at the 0.05 significance level. The mean for the 0.12 mg/kg PAPP dose was not statistically significantly greater than the control mean. The random effect for animal-to-animal variability ($p = 0.001$) and dose sequence effect ($p = 0.020$) were significant for time to peak heart rate. Further investigation showed that while the ln-transformed time to peak heart rate was lower for the second experiment, particularly in the high dose group, no trend over the course of the four treatments was evident. None of the analysis of variance effects was significant at the 0.05 level for the peak shift from baseline heart rate.

The effect of PAPP dose was statistically significant for time to peak respiratory rate ($p < 0.001$). The average of the ln-transformed time to peak was statistically greater at each PAPP dose compared to the 0 mg/kg control. The dose effect was not statistically significant for the peak shift from baseline respiratory rate. The random effect for animal-to-animal variability was significant for both time to peak ($p = 0.028$) and peak shift from baseline respiratory rate ($p = 0.001$). The dose sequence effect was not significant for these endpoints.

The physiological progression of events for animals in Phase III, across all doses, was generally as follows:

(1) RA was followed by peaks of both total and free plasma CN^-.

TABLE 23. PHASE III-SUMMARY OF THE STATISTICAL ANALYSIS OF THE EFFECTS OF PAPP DOSE, ANIMAL-TO-ANIMAL VARIABILITY, AND DOSING SEQUENCE ON PHYSIOLOGIC AND CN^- PARAMETERS

		PAPP Dose Effect							
		PAPP Dose (mg/kg)					Animal Variability[a]		Dosing Sequence[b]
Parameter	Endpoint	0	0.12	0.17	0.37	p-value	σ_A	p-value	p-value
Heart Rate	Time to Peak (min) [c]	1.29	1.54	1.89[d]	3.76[d]	<0.001	0.22	0.001	0.020
	Peak Shift from Baseline (beats per minute)	109.26	93.61	109.88	97.65	0.909	32.08	0.066	0.483
Respiratory Rate	Time to Peak (min)[c]	1.39	2.03[d]	2.60[d]	5.41[d]	<0.001	0.15	0.028	0.363
	Peak Shift from baseline (breaths per minute) (μg/mL)	68.52	74.00	75.10	68.66	0.871	21.78	0.001	0.212
Free Plasma CN^-	Time to Peak (min)[c]	2.60	3.98[d]	4.62[d]	9.33[d]	<0.001	0.15	0.003	0.434
	Peak (μg/mL)	1.30	0.76[f]	0.61[f]	0.38[f]	<0.001	0.21	0.044	<0.001
Total Blood CN^-	Average Level after Respiratory Arrest[c]	6.60	13.61[d]	16.93[d]	36.91[d]	<0.001	0.06	0.198	0.129
	Uptake ((μg/mL)/min)	1.80	3.93[e]	4.54[e]	4.62[e]	<0.001	0.50	0.027	0.014

[a] σ_A = Estimate of the animal-to-animal standard deviation, and p-value = Observed significance level for the animal-to-animal variance component.
[b] p-value for the significance of the dosing sequence.
[c] Statistical analysis was conducted on ln-transformed values for this endpoint. Estimated geometric means are reported for each PAPP dose. The animal-to-animal ln standard deviation estimate is reported.
[d] Average of ln-transformed values was significantly greater than that observed for the 0 mg/kg control dose at the 0.05 significance level.
[e] Average value was statistically greater than that observed for the 0 mg/kg control dose at the 0.05 significance level.
[f] Average value was statistically less than that observed for the 0 mg/kg control dose at the 0.05 significance level.

(2) The average TRA increased with increasing dosages of PAPP, and was particularly longer for the 0.37 mg/kg dose group compared to the lower PAPP dosage groups.

(3) Once NaCN infusion began, an effect on the respiratory parameters was observed within 18-90 seconds, depending on PAPP dosage.

(4) The initial respiratory effect observed was a slight decrease in rate, with elongation of the inspiratory waveform (increase in time of inspiration) and slight decrease in respiratory waveform amplitude, followed by an immediate increase in respiratory rate. This increase in respiratory rate also was characterized by an increase in amplitude of the waveform. For most animals, amplitude and rate of respiratory waves then gradually decreased until RA occurred. Some animals took periodic gasps as breathing slowed, while others would appear to stop breathing, and then take periodic gasps until RA occurred.

(5) An increase in the heart rate was observed shortly (at lower PAPP doses almost instantaneously) after the increase in respiratory rate began.

(6) Across dosages, peak heart rates were followed by peak respiratory rates.

(7) Across the dosages, the following events were typically observed on ECG tracings during NaCN infusions. Initially, a sinus tachycardia was observed, and progressed to a sinus bradycardia as RA approached. As CN^- toxicity was observed, the ECG tracing resembled a typical hypoxic tracing with increasing T-wave amplitude and elevation of the ST segment. At or shortly after RA, a greater severity of arrhythmias (severe bradycardia, heart block of various degrees, including complete heart block, ventricular tachycardia) were observed. In addition, a reduction in QRS amplitude, premature ventricular complexes (PVCs), and inverted QRS complexes were observed.

(8) The animal-to-animal variability noted in TRA and percent MHb parameters was influenced by the individual animal's body weight and pharmacokinetics. Each animal varies in its ability to absorb, metabolize, and eliminate various compounds, and to form MHb. The animal-to-animal variability for these parameters appears to increase with increasing PAPP dosage.

CN^- Parameters: The effect of PAPP dose on total blood CN^- was analyzed using the average level after RA and the rate of uptake during NaCN infusion. The effect of PAPP dose on free CN^- was analyzed using the time to peak and peak level of free CN^- in plasma. Descriptive statistics for these endpoints are presented in Table H-4. Total CN^- in blood

samples and free CN^- in plasma samples taken during and after the NaCN infusion are plotted against time for each animal in Figures H-24 through H-39.

Analysis of variance results are summarized in Table 23. ANOVA models were fitted to: (1) uptake and average total blood CN^- levels after RA, (2) peak level and time to peak level of free plasma CN^-. PAPP dose and dosing sequence were utilized as explanatory variables and a random effect was included for animal-to-animal variability in these models. Because the average level of total blood CN^- and time to peak free plasma CN^- appeared to increase in variability with PAPP dose, ln-transformed values were analyzed for these endpoints. Geometric means at each PAPP dose and the estimated animal-to-animal standard deviation of the ln-transformed values are presented in Table 24 for the average level of total blood CN^- and for time to peak free plasma CN^-. Ln-transformed average levels and uptake of total blood CN^- are plotted against PAPP dose in Figures H-40 and H-41. Figures H-42 and H-43 present plots of ln-transformed time to peak and peak levels of free plasma CN^-.

The effects of PAPP dose on the average total CN^- level after RA and on total CN^- uptake were statistically significant. The means at each PAPP dose level were significantly greater than the control dose mean for both the ln-transformed average levels and the rate of uptake. Animal-to-animal variability and the effect of dosing sequence were both significant for total CN^- uptake. Further investigation showed that while the rate of uptake was greater for the second experiment, particularly in the high dose group, no trend over the course of the four treatments was observed. Neither animal-to-animal variability nor dose sequence significantly affected the average level of total blood CN^- after RA.

The effect of PAPP dose on the peak and ln-transformed time to peak free CN^- was statistically significant . The average peak free CN^- at each PAPP dose was significantly less than the average at the 0 mg/kg dose. The time to peak was significantly greater at each PAPP dose compared to the control dose. Animal-to-animal variability was significant for both the peak free plasma CN^- and the time to peak. The effect of dosing sequence was significant for the free CN^- peak, but not for the time to peak. Further investigation showed that the high dose peaks were consistent across the experiment, but that the peaks in the other dose groups were lower and less variable in the first dosing than at later dosings. This may be a

phenomenon worthy of additional experimentation, or it could be a consequence of the discrete sampling times.

Phases IV and VIII - Between Phase IV and VIII experiments, there was an approximately one month washout. The experiments, however, are discussed together.

Tables I-1 (Appendix I) and J-1 (Appendix J) present the actual MHb levels, TRAs, and NaCN infusion doses for each pretreatment compound or the vehicle for each animal on each dosing day for Phases IV and VIII, respectively. To facilitate comparisons among PAPP, WR242511, and PAHP-dosed animals, statistical analyses were conducted on the combined data from Phases IV and VIII. Descriptive statistics for TRA, infused NaCN dose, and percent MHb for each pretreatment of Phases IV and VIII are presented in Table 24. Because NaCN infusion for PAHP and WR242511 experiments was conducted once the measured MHb level fell within a specified range, the standard deviation of percent MHb does not reflect the variability in uptake of these compounds.

TABLE 24. PHASE IV AND VIII EXPERIMENTS-DESCRIPTIVE STATISTICS FOR TIME TO RESPIRATORY ARREST, INFUSED NaCN DOSE, AND PERCENT MHb

Endpoint	Treatment	Mean	Standard Deviation	Minimum	Maximum
Time to Respiratory Arrest (min)	Control (Phase IV)	2.51	0.37	2.05	3.25
	PAPP	4.97	0.85	3.37	6.40
	WR242511	5.31	1.55	3.62	8.87
	PAHP	5.59	1.47	4.17	9.08
	Control (Phase VIII)	2.92	0.50	2.37	3.85
Infused NaCN Dose (mg/kg)	Control (Phase IV)	1.54	0.24	1.24	1.85
	PAPP	2.99	0.54	2.24	3.78
	WR242511	3.49	1.02	2.07	5.83
	PAHP	3.54	0.90	2.55	5.44
	Control (Phase VIII)	1.80	0.37	1.02	2.33
Percent MHb (%)	Control (Phase IV)	0.00	0.00	0.00	0.00
	PAPP [a]	4.92	1.26	3.10	7.00
	WR242511[a]	5.00	0.94	3.40	6.10
	PAHP [a]	4.92	1.11	3.00	6.50
	Control (Phase VIII)	0.00	0.00	0.00	0.00

[a] PAHP, PAPP, and WR242511 experiments were designed to induce MHb levels of approximately 5 percent.

Descriptive statistics for heart rate, respiratory rate, and total blood CN^- endpoints for Phase IV and Phase VIII experiments are displayed in Table 25. Moving average heart rate is plotted over time for each animal in Figures I-1 through I-9 for Phase IV experiments and in Figures J-1 through J-9 for Phase VIII experiments. Moving average respiratory rate is plotted over time for each animal in Phase IV experiments in Figures I-10 through I-18 and in Figures J-10 through J-18 for Phase VIII experiments. The events for animals in these phases, across compounds, were similar to those reported for Phase III:

(1) The average time from the start of NaCN infusion to RA was 4.97 minutes for PAPP, 5.31 minutes for WR242511, and 5.59 min for PAHP, while the average MHb level when NaCN infusion began was 4.92, 5.00, and 4.92 percent, respectively. For control animals, the average time from start of NaCN infusion to RA was approximately 2.5 minutes in Phase IV experiments and 2.92 in Phase VIII experiments.

(2) After NaCN infusion had begun, an effect on respiratory parameters was observed within 20-45 sec, depending upon MHb levels and whether the vehicle control or a MHb-forming compound was dosed.

(3) The same relationship between respiratory and heart rates reported for Phase III, Physiologic Parameters was observed across compounds.

(4) Observations on ECG tracings reported for Phase III, Physiologic Parameters were similar for each compound in this phase.

(5) The animal-to-animal variability noted in TRA and the infused NaCN dose was influenced by the individual animal's body weight and pharmacokinetics. Each animal varies in its ability to absorb, metabolize, and eliminate various compounds, and in the ability to form MHb.

Figures I-19 through I-27 present graphs of percent MHb and total blood CN^- plotted over time for each animal in Phases IV and VIII. It is evident from these figures that in vehicle control experiments of Phase VIII, total blood CN^- levels were slightly greater than those observed in Phase IV animals.

TABLE 25. PHASE IV AND VIII EXPERIMENTS-DESCRIPTIVE STATISTICS FOR PHYSIOLOGIC AND TOTAL BLOOD CN^- PARAMETERS FOR THREE METHEMOGLOBIN FORMERS

Parameter	Endpoint	Treatment	Mean	Standard Deviation	Minimum	Maximum
Heart Rate	Time to Peak (min)	Control (Phase IV)	1.34	0.25	1.00	1.75
		PAPP	2.03	0.64	1.17	3.25
		WR242511	2.27	1.20	1.58	5.42
		PAHP	2.30	0.94	1.50	4.00
		Control (Phase VIII)	1.24	0.18	1.00	1.50
	Peak Shift from Baseline (beats per minute)	Control (Phase IV)	67.01	48.87	11.41	165.73
		PAPP	78.52	29.84	49.86	136.27
		WR242511	79.56	30.30	22.90	112.78
		PAHP	76.14	36.55	17.77	120.77
		Control (Phase VIII)	63.00	33.45	16.19	109.69
Respiratory Rate	Time to Peak (min)	Control (Phase IV)	1.43	0.26	1.17	1.75
		PAPP	2.57	0.54	1.67	3.50
		WR242511	2.30	0.78	1.50	3.75
		PAHP	2.55	0.81	1.83	4.58
		Control (Phase VIII)	1.45	0.34	0.83	1.92
	Peak Shift from Baseline (breaths per minute)	Control (Phase IV)	49.88	18.85	19.79	70.27
		PAPP	49.23	12.49	23.57	63.21
		WR242511	58.04	11.36	43.29	81.89
		PAHP	64.41	13.13	43.57	89.19
		Control (Phase VIII)	69.66	18.20	39.25	105.22
Total NaCN	Average Level after Respiratory Arrest (μg/mL)	Control (Phase IV)	6.44	0.80	5.24	7.75
		PAPP	17.74	3.86	9.90	22.40
		WR242511	18.79	4.20	13.80	27.38
		PAHP	17.95	4.33	11.00	25.02
		Control (Phase VIII)	7.91	0.85	6.88	9.33
	Uptake ((μg/mL)/min)	Control (Phase IV)	1.69	0.34	1.03	2.20
		PAPP	3.82	0.94	2.53	5.27
		WR242511	3.88	0.57	3.20	5.02
		PAHP	3.62	0.74	2.49	4.44
		Control (Phase VIII)	1.58	0.32	0.97	2.00

Results of the analyses of variance are presented in Table 26 for Phases IV and VIII data. Because the variability was larger for the PAPP, WR242511, and PAHP experiments than for the vehicle experiments, analyses were conducted on ln-transformed values for TRA and infused NaCN dose. Phase IV and Phase VIII control means were not significantly different for TRA, infused NaCN dose, or percent MHb. While the PAPP, WR242511, and PAHP treatment compounds had significant effects on TRA, infused NaCN dose, and percent MHb compared to the Phase IV and Phase VIII controls, they were not significantly different from each other in any of the three endpoints. The variability between animals was statistically significant for TRA and infused NaCN dose. Although mean TRA and infused NaCN dose were larger for vehicle experiments conducted in Phase VIII when compared to those conducted in Phase IV, they were not statistically different based on analysis of variance results.

TABLE 26. PHASES IV AND VIII-ANALYSIS OF VARIANCE RESULTS FOR TIME TO RESPIRATORY ARREST, INFUSED NaCN DOSE, AND PERCENT MHb

Endpoint	Effects Included in Analysis of Variance: Animal p-value	Effects Included in Analysis of Variance: Treatment p-value	Results of Multiple Comparisons Across Treatment Groups
Time to Respiratory Arrest [a] (min)	<0.001	<0.001	PAPP = WR242511 = PAHP Control (Phase IV) = Control (Phase VIII)
Infused NaCN [a] (mg/kg)	<0.001	<0.001	PAPP = WR242511 = PAHP Control (Phase IV) = Control (Phase VIII)
Percent [b] MHb(%)	0.374	<0.001	PAPP = WR242511 = PAHP Control (Phase IV) = Control (Phase VIII)

[a] Analysis of variance conducted on ln-transformed data.

[b] PAHP, PAPP and WR242511 experiments were designed to induce MHb levels of approximately 5 percent.

Parameter estimates and results of the treatment comparisons for linear regression models fitted to TRA and infused NaCN dose for the PAPP, WR242511, and PAHP data are presented in Table 27. Figures 5 and 6 display the fitted regression lines and individual

observations of TRA and infused NaCN dose plotted against percent MHb for the three pretreatment compounds. No significant differences were detected when comparing estimated slopes and intercepts of TRA and infused NaCN dose versus MHb levels for PAPP, WR242511, and PAHP treated animals.

TABLE 27. PHASES IV AND VIII-SUMMARY OF REGRESSION OF TIME TO RESPIRATORY ARREST AND INFUSED NaCN DOSE VERSUS PERCENT MHb FOR PAPP, WR242511, AND PAHP

	Model Estimates				Treatment Comparisons		
Response	Treatment	Intercept	Slope	Estimated Response at 5% MHb	Treatment Groups	Intercept Comparison p-value	Slope Comparison p-value
Time to Respiratory Arrest (min)	PAPP	1.92	0.62	5.02	PAPP, WR242511	0.326	0.222
	WR242511	0.54	0.95	5.31	PAPP, PAHP	0.892	0.819
	PAHP	2.15	0.70	5.64	WR242511, PAHP	0.470	0.561
Infused NaCN Dose (mg/kg)	PAPP	1.17	0.37	3.02	PAPP, WR242511	0.583	0.302
	WR242511	0.63	0.57	3.49	PAPP, PAHP	0.233	0.051
	PAHP	0.23	0.67	3.59	WR242511, PAHP	0.755	0.692

Phase V - Pretreatment Dose Selection - PAPP was the pretreatment of choice for Phase V because PAPP was easy to administer, has a short half life, would create the target MHb level in a short period of time (minutes vs hours), and responses were less variable than for other pretreatment compounds. Phase III results indicated that the average infused NaCN dose resulting in RA for the eight animals at the 0 mg/kg control dose was 1.5 mg/kg. Thus, the 2x AvTRA dose was 3.0 mg NaCN/kg BW. Phase III data were used to estimate the MHb level that would protect 95 percent of animals against a 2x AvTRA challenge. Linear regressions between percent MHb and infused NaCN dose were used to determine the percent

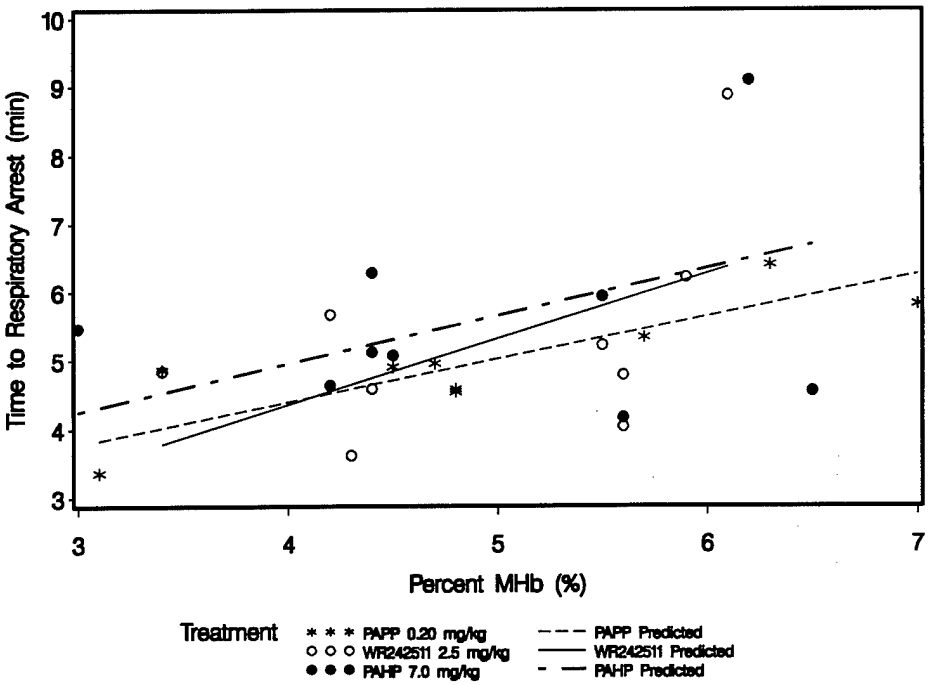

FIGURE 5. PHASES IV AND VIII-TIME TO RESPIRATORY ARREST PLOTTED AGAINST PERCENT MHb. OBSERVED VALUES OVERLAID ON REGRESSION LINES FOR PAPP, WR242511, AND PAHP

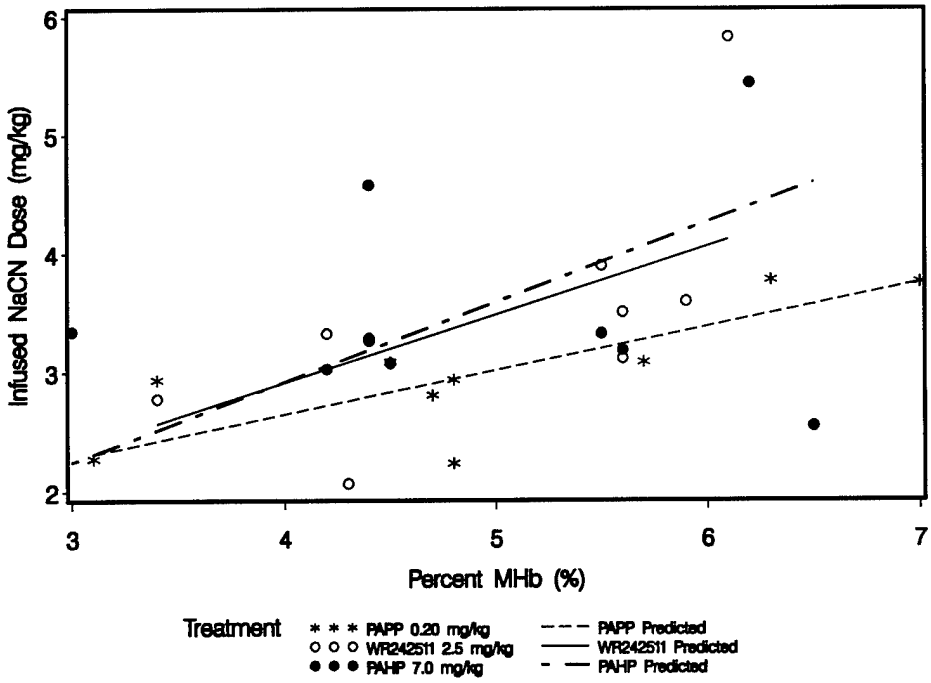

FIGURE 6. PHASES IV AND VIII-INFUSED NaCN DOSE PLOTTED AGAINST PERCENT MHb. OBSERVED VALUES OVERLAID ON REGRESSION LINES FOR PAPP, WR242511, AND PAHP.

MHb required to protect against 2x AvTRA for each animal. The mean estimated MHb level was 5.4 percent with a 95 percent upper confidence limit of 6.4 percent. Therefore, a target MHb level of 6.5 percent was selected.

Based on linear regression between PAPP dose and percent MHb using Phase II data, a PAPP dose of 0.25 mg/kg was estimated to produce 6.5 percent peak MHb on average. To ensure that all animals would reach at least 6.5 percent, a PAPP dose of 0.3 mg/kg was selected.

Table K-1 in Appendix K presents the measured times of NaCN infusion, calculated and analyzed NaCN doses, and actual MHb levels measured when NaCN infusion was initiated. Descriptive statistics for primary and secondary endpoints are displayed in Table K-2. Moving average heart rate is plotted over time for each animal in Figures K-1 through K-10. Computer-collected heart rate data were incomplete for Animal HFZADG because a cable from the recorder to the PO-NE-MAH computer became loose (Analog data were recorded on the chart recorder.). Moving average respiratory rate is plotted over time for each animal in Figures K-11 through K-20. The physiological progression of events for animals in Phase V was generally as follows:

(1) After NaCN infusion began, an effect on respiratory parameters was observed within 30-35 seconds.

(2) The respiratory effects observed on tracings were the same as those reported in Phases III and IV except that the amplitude and rate of respiratory waves were elevated or beginning to decrease (depending on the dose of NaCN infused) when the NaCN infusion was terminated. None of the animals ceased breathing during NaCN infusion.

(3) Across the animals, peak heart rates were followed by peak respiratory rates.

(4) The following events were typically observed on the ECG tracings during NaCN infusion. Early in NaCN infusion, a sinus tachycardia was observed, but heart rate was beginning to slow when NaCN infusion ceased. As the toxic effects of CN^- were observed, the ECG resembled a typical hypoxic tracing with increasing T-wave amplitude and elevation of the ST segment. The degree of T-wave elevation and S-T segment elevation in this phase, however, was not as pronounced

as in earlier phases when RA did occur. Major arrhythmias were not observed. In general, sinus tachycardia, typical hypoxic tracings with T-wave and S-T segment elevations, and reduction in amplitude of the QRS complexes were the most severe events on ECG tracings.

Percent MHb and total blood CN^- are plotted over time for each animal in Figures K-21 through K-30. The average MHb level of 6.6 percent and the average infused NaCN dose of 3.0 mg/kg did not differ significantly from the targeted values. All ten animals were protected from a 2x AvTRA challenge.

4.0 CONCLUSIONS

Feasibility Study (Phase I): Results of the repeated dosing protocol indicated that its use in this study would not impact study parameters. Repeated dosing did not appear to significantly impact TRA, baseline heart rates, or respiratory rates. There were no apparent trends for changes in heart rate, respiratory rate, total or free CN^- levels, or MHb levels as a result of repeated testing. Baseline Hb levels did appear to be affected by repeated experimentation. Although baseline Hb values within the same dog were significantly lower in subsequent experiments, hematocrit readings, Hb levels, and overall physical evaluations over the 4 weeks suggested that each animal was in good physical condition and none exhibited adverse effects. The hematocrit and Hb values remained within published normal limits.

Single Dose Pharmacodynamic Studies (Phases II and VII): Phase II - PAPP: Time course plots of the modelled PAPP dose curves overlaid on the observed percent MHb for each animal indicated that the model fitted the data well. The PAPP data indicated a dose-response effect between peak percent MHb and time to peak. The PAPP doses estimated, using the fitted model data, to produce 2.5, 5, and 10 percent MHb were 0.12, 0.17, and 0.37 mg PAPP/kg BW, respectively, and the predicted time to peak for each estimated PAPP dose was 28, 38, and 56 min, respectively. Animal-to-animal variability was significant for both modelled and empiric peak percent MHb. The between animal variability may be explained by the recognized variation in rates of Hb oxidation and by individual biological

variation. The animal-to-animal variability in time to peak was not significant. There were no apparent PAPP dosing sequence effects on any of the parameters.

WR242511: Time course plots of the modelled WR242511 dose curves overlaid on the observed percent MHb for each animal suggested a good fit. The relationship between peak percent MHb and WR242511 dose was nonlinear, and therefore linear dose-response models were not fitted to the data.

Figure 7 displays the duration for each dog that the percent MHb ranged between approximately 6.5 and 3.5 percent based on the modelled time course data for a 2.5 mg/kg WR242511 dose. The plot suggests that peak levels of MHb occur approximately 3 to 6 days following administration of this dose of WR242511, and that the MHb level remains at approximately 5 percent for a 3 to 4 day period.

Phase VII - Oral dosing of PAHP dissolved in PEG200 and placed within a gelatin capsule showed greater variability in peak percent MHb data than orally administered WR242511. Increased variability in percent MHb following PAHP dosing made it difficult to predict MHb levels following an oral dose.

Multiple Dose Pharmacodynamic Study (Phase VI): WR242511 and PAHP: WR242511 was easier to administer, was a more stable test article, required less frequent dosing, and was more predictable in MHb levels produced when compared to PAHP. PAHP administration required dosing three times a day and the resultant MHb levels varied greatly between doses. Steady state levels were generally reached after 4 to 7 doses of either test compound. MHb levels following repeated dosings of WR242511 were more stable and more predictable than those observed following repeated dosings of PAHP.

WR242511: Most animals in the WR242511 treatment group reached 95 percent of the SS percent MHb during the fourth dose period (6 to 8 days following the first dose) and stayed near that level for the remainder of the experiment.

The weighted mean SS was 12.0 percent MHb, although the estimated SS averages ranged between 8.6 and 15.9 percent MHb for individual animals. The estimated time to 95 percent (t_{95}) of SS ranged between 7.2 and 16.3 days with a weighted average of 8.1 days

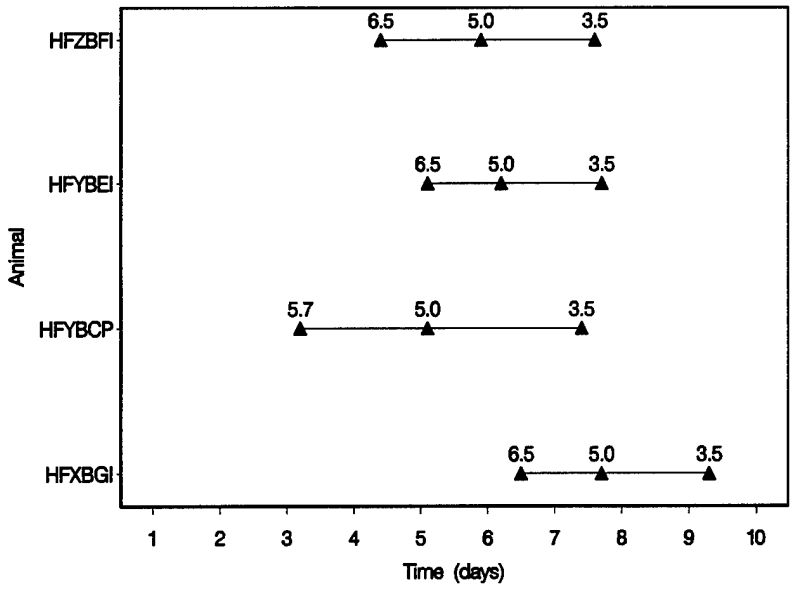

FIGURE 7. PHASE II-DURATION OF TIME PERCENT METHEMOGLOBIN LEVEL RANGED FROM 6.5 TO 3.5 PERCENT FOLLOWING PEAK BASED ON MODELLED PERCENT METHEMOGLOBIN TIME COURSE DATA FOR WR242511

(approximately the fourth dose period).

PAHP: For PAHP experiments the weighted mean SS trough was 9.4 percent MHb. Peak MHb levels greater than 30 percent were observed in five of the animals. The t_{95} ranged between 2.1 and 19.9 days, with a weighted average of 2.4 days (approximately the seventh dose period).

Efficacy Studies (Phases III, IV, V, and VIII): Phase III - Experiments were conducted to assess the efficacy of PAPP at doses predicted to produce 0, 2.5, 5, and 10 percent MHb. The following conclusions were based on the statistical analyses of TRA, NaCN dose infused, and percent MHb.

(1) Average time to RA for each PAPP-dosed group (0.12, 0.17, and 0.37 mg/kg PAPP) was significantly greater than that observed for the 0 mg/kg control group. A statistically significant relationship was observed between percent MHb and ln-transformed time to RA.

(2) Average amount of infused NaCN for each PAPP-dosed group was significantly greater than that observed for the control group. A statistically significant relationship was observed between percent MHb and ln-transformed NaCN dose infused.

(3) Average percent MHb for each PAPP-dosed group was significantly greater than that observed for the control group.

(4) Repeated NaCN infusions did not appear to have a significant impact on TRA, infused NaCN dose, or percent MHb produced. The animal-to-animal variability was statistically significant for TRA and percent MHb.

The effect of PAPP dose on respiratory rate, heart rate, total blood CN^- and free plasma CN^- were investigated. The following conclusions are based on the statistical analyses of the physiologic and NaCN response parameters.

(1) Average times to peak heart rate following 0.17 or 0.37 mg PAPP/kg BW and NaCN infusion were significantly greater than those observed for the control group. The magnitude of the peak shift from baseline heart rate did not appear to be related to PAPP dose.

(2) Average time to peak respiratory rate for each PAPP-dosed group was significantly greater than that observed for the control group. The magnitude of the peak shift from baseline respiratory rate did not appear to be related to PAPP dose.

(3) Average total blood CN^- following RA for each PAPP-dosed group was significantly greater than that observed for the control group. The average uptake rate of total blood CN^- for each PAPP-dosed group was significantly greater than that observed for the control group.

(4) Average time to peak free plasma CN^- for each PAPP-dosed group was significantly greater than that observed for the control group. Average peak level of free CN^- for each dose of PAPP was significantly less than that observed for the control group.

(5) The animal-to-animal variability was statistically significant for time to peak respiratory and heart rates, peak shift from baseline respiratory rate, uptake rate of total blood CN^-, and time to peak and peak levels of free plasma CN^-. Variability due to repeated dosing was statistically significant for time to peak heart rate, uptake rate of total blood CN^-, and peak levels of free plasma CN^-.

The effect of PAPP across doses was to increase the TRA, the amount of NaCN infused until RA, and the percent MHb. In addition, the effect of PAPP dosing was to delay the initiation of increased heart rate and respiratory rate, to attenuate the rate of increase of both heart rate and respiratory rate, and to prolong the times to peak heart rate, peak respiratory rate, and peak free plasma CN^-. This was most dramatic when comparing the zero PAPP dose to the 0.37 mg PAPP/kg BW dose.

The fitted linear regression between percent MHb and infused NaCN dose was utilized to estimate the percent MHb required for protection from a 2x AvTRA NaCN dose. The average estimated percent MHb for such protection was 5.4 percent with a 95 percent upper prediction bound of 6.0 percent. Based on this information and pharmacodynamics data collected in Phase II, a dose of 0.3 mg PAPP/kg BW was recommended to produce approximately 6.5 percent MHb to protect against a 2x AvTRA NaCN challenge.

Phases IV and VIII - PAPP, WR242511, and PAHP pretreatment regimens were effective in mitigating the effects of NaCN intoxication when compared to controls. No significant differences in efficacy were observed among the three compounds. The mitigating effect appears to be related only to the induced MHb level. The relationships of TRA and infused NaCN dose with percent MHb appear to be similar for all three compounds.

Phase V - The PAPP pretreatment regimen predicted to produce 6.5 percent MHb was effective in protecting all of the animals against the effects of a 2x AvTRA challenge. The lower 95 percent confidence limit of the proportion of animals protected under these conditions is 74 percent.

5.0 DISCUSSION

A moderate plane of anesthesia was indicated by slight to no corneal (eye blink) reflex, minimal to no rear limb toe pinch reflex (somatic muscular relaxation moderate to marked), normal to slightly dilated pupils, and normal to decreased rate and depth of respiration. Most animals were within Plane II of Stage III surgical anesthesia at the time of NaCN infusion. Some animals required additional doses of anesthetic between initial induction and initiation of NaCN infusion. Depth of anesthesia may have contributed to the inter- and intra-animal

variability observed for some parameters. Analyses indicated that repeated anesthesia produced no significant changes in the physiological end-points monitored.

The ultra-short-acting barbiturate used for induction of anesthesia from September 24, 1993 through February 21, 1994 (Phases I, II, and III) was thiamylal sodium. Thiamylal sodium became commercially unavailable, and throughout the remainder of the study (Phases III, IV, V, and VIII), sodium thiopental was used. Pentobarbital sodium was used to maintain anesthesia. Table 28 displays the phases (III, IV, VIII, and V), animals, volume of additional sodium pentobarbital administered, and the time prior to NaCN infusion the additional anesthetic was injected.

TABLE 28. ADMINISTRATION OF ADDITIONAL PENTOBARBITAL SODIUM AFTER OR DURING PRETREATMENT DOSING

Phase	Animal Number	Period	Amount of sodium pentobarbital administered (mL)	Time prior to NaCN infusion (min)
III	HFYAZF	1	0.5	21
			0.5	16
		3	0.5	9
		4	0.75	2
	HFYAIH	1	0.5	20
		4	0.5	25
			0.4	7
	HFYBKC	2	0.5	17
	HFYBFM	2	1.0	31
		4	0.5	4
	HFZADG	3	0.25	13
	HFZADD	3	0.75	52
			0.25	25
			0.5	4
		4	0.5	17

TABLE 28.
(Continued)

Phase	Animal Number	Period	Amount of sodium pentobarbital administered (mL)	Time prior to NaCN infusion (min)
	HFZBDC	4	0.75	27
			0.5	25
			0.5	20
			0.5	7
	HFXAEN	4	0.7	3
IV	HFYAGL	1	0.5	12
		2	0.5	37
			0.5	3
	HFYAWJ	1	0.4	1
	HFZAGX	1	0.5	34
	HFYAJM	1	1.0	8
			0.5	3
	HFZBAV	1	0.25	12
	HFZAHB	2	0.4	1
	HFYATS	2	0.5	14
VIII	HFZANH	1	0.5	3
		2	1.0	53
			0.5	42
			0.5	5
	HFYAGL	1	0.5	4
	HFZBAV	1	0.5	15
	HFZAHB	1	0.5	16
			0.5	11
V	HFZAGX	1	1.0	9

Following 30 sec of RA induced by NaCN, hydroxylamine was given IV, and additional symptomatic therapy was given in Phase I and early in Phases III and IV. Additional therapy may not have been required, and therefore was administered only on an as-needed basis. The most commonly used adjunct treatments were lidocaine for treatment of ventricular arrhythmias, atropine sulfate for vagal slowing of the heart, and methylene blue for MHb levels greater than 50 percent. Table 29 summarizes additional therapies administered. Over the course of the study, clinical evaluation of animals that received additional therapy indicated that this therapy may not have been necessary. Animals showing similar signs (i.e., arrhythmias, heart block, severe bradycardia, severe methemoglobinemia) recovered within several min without additional treatment, and recovery time appeared to be reduced. Initially, oxygen was administered approximately 3 min after hydroxylamine injection in NaCN-infused animals, but the Study Director changed this to routinely administering room air by Ambu® bag to recovering animals. The degree of arrhythmias and duration appeared to be reduced using room air as opposed to oxygen.

TABLE 29. ADDITIONAL THERAPIES ADMINISTERED

Phase	Animal ID	Date Administered	Drug and Volume
I	HFXAYK	12-22-93	0.3 mL Atropine Sulfate
III	HFZADG	01-18-94	2.1 mL Methylene Blue
	HFZBDC	01-19-94	1.0 mL Atropine Sulfate
	HFYBKC	01-20-94	0.5 mL Lidocaine
	HFYAZF	01-21-94	0.5 mL Atropine Sulfate
	HFYAIH	01-27-94	1.0 mL Methylene Blue
	HFZBDC	02-15-94	0.6 mL Lidocaine
	HFYBFM	02-23-94	0.13 mL Atropine Sulfate
			0.5 mL Lidocaine
IV	HFYAWJ	04-05-94	0.5 mL Lidocaine
VIII	N/A	N/A	N/A
V	N/A	N/A	N/A

Feasibility Study (Phase I): The effect of repeated experimentation on measured baseline physiological parameters was significant for Hb levels only. The observed significance resulted presumably from multiple blood sample collection (ranging from 15 to 18 samples of 1-3 mL for any one experiment). Over the four-week period of repeated experimentation, baseline hematocrit readings decreased over time. However, these readings were within the reported normal range of 37 to 55 percent.[16] Dehydration of the animals was not observed clinically. Hematocrit readings of study animals were started within 72 hours of pretreatment dosing or periodically during blood sample collection periods, depending upon the phase.

Single Dose Pharmacodynamic Studies (Phase II and VII): Phase II - PAPP: Animals were anesthetized prior to administering PAPP to simulate the method and route of PAPP administration used in Phases III, IV, and V. Data collected and used to predict doses and MHb levels for the various phases correlated well with the fitted model.

WR242511: On the fourth day (Friday, October 29, 1993) after dosing in the first week of the experiment, the MHb values were unusually high for each animal and each dose. These values were used in smoothing the percent MHb data for the fitted models, but were not used in calculating the empiric means. Review of the instrumentation calibration data for that day did not offer an explanation for these abnormally high readings. However, review of the daily sample data indicated that these samples may have been read in the "human mode" and not the "dog mode". This would account for the higher MHb values recorded.

Phase VII - Pilot Study: PAHP was slightly soluble in water, but not sufficiently to allow dosing in capsules. Therefore, PAHP was dissolved in PEG200 for gelatin capsule administration in Phases VII and VIII. Animals were dosed per os similarly to WR242511 dosed animals in Phase II.

Study: Means of modelled peak percent MHb at 2.5 mg WR242511/kg BW and 5.0 mg PAHP/kg BW were similar. Although the means were similar, the standard deviation of peak percent MHb at 5 mg PAHP/kg BW was nearly twice that observed at 2.5 mg WR242511/kg BW. The increased variability in peak percent MHb following oral administration of PAHP compared to that observed for oral administration of WR242511 made it more difficult to

accurately predict percent MHb levels resulting from PAHP dosing. Therefore, it was necessary to monitor percent MHb levels following PAHP dosing and to administer the NaCN once the MHb levels fell within a specified range about 5 percent. The increased variability observed in MHb following oral administration of PAHP is probably the result of the route of administration and biological variability. Therefore, the procedures used in Phase VIII experiments were:

(1) A fixed dose of PAHP was administered. If peak levels of 5 percent MHb were not attained, a higher dosage could be given.

(2) Percent MHb levels were monitored following PAHP dosing.

(3) NaCN infusion was initiated once the percent MHb level fell to a range about 5 percent.

(4) The range of MHb levels employed in Phase VIII for PAHP was the same as that used in Phase IV for WR242511, i.e., 3 to 7 percent MHb.

Multiple Dose Pharmacodynamic Study (Phase VI): WR242511: Most blood samples were collected within the targeted time for each animal. On study day 11 (June 18, 1994), blood samples for animals HFZADP, HFXBAD, HFXAYK, and HFXAYH were approximately 28, 25, 22, and 19 minutes late, respectively. Blood samples from these animals had been analyzed erroneously using the "human mode" on the hemoximeter. Additional blood samples were drawn and analyzed using the "dog mode". This did not affect the integrity of the study since the data collected were used in a dose-response versus time curve and the actual draw times were used in the model.

On study day 8 (June 15, 1994), animal HFZADI vomited approximately 40 min after dosing. The dose was not re-administered. Vomiting may have resulted from drug administration on an empty stomach. On study day 9 (June 16, 1994), animal HFZADI vomited approximately 24 hr after dosing, and all six animals vomited approximately 30 hr after dosing (1430 observation). These animals appeared in good health and alert, and were

eating and drinking normally throughout the study. An additional study may be necessary to determine the effect of this compound on the gastrointestinal tract.

PAHP: Animal HFXBGI was dosed at 00:26 of study day 3. Approximately 19 min after dosing, a portion of the dissolved capsule and vomitus were observed in the animal's cage, and the Study Director was notified. Based on the technician's observation and comparison of MHb data from previous dosings to current results in this animal, the Study Director decided not to redose in order to prevent excessively high MHb levels. A memo was written to the study file and the Contracting Officer's Representative (COR) notified of this event.

On study day 8 (July 25, 1994), HFZAYM collapsed during exercise. The technician notified the Study Director that the animal had collapsed, his eyes had "rolled back", and an extremely rapid pulse was palpated. After approximately one min, the animal recovered and appeared normal. The Study Director restricted exercise for the animals on this phase and continued the dosing regimen for the remainder of the dosing schedule until the animals were evaluated the next morning. The technicians were instructed to closely monitor the animals and notify the Study Director of any abnormal observations.

On study day 9 (July 26, 1994), hematocrit readings and MHb levels for each animal were reviewed, and the collapse of animal HFZAYM was evaluated by the Study Director and COR. Low hematocrit readings (less than 35 percent) and low Hb levels coupled with high MHb levels (greater than 18 percent) resulted in termination of the study for animals HFXAGU and HFZAYM. The remainder of the animals were dosed at 1600 to 1626 on July 26, 1994, and the blood collections for the limited pharmacokinetic portion of the study were performed prior to study termination.

Efficacy Studies (Phases III, IV, V, and VIII): Phase III - All animals recovered from experiments without any apparent adverse effects, whether additional therapy was administered or not. For the remainder of the study when NaCN infusion was performed, respiratory assistance and extra therapy were administered on an as-needed basis.

Phases IV and VIII - The pretreatment dosing protocol for each compound and the time delay prior to infusion of NaCN varied for each compound. PAPP was administered IV and

NaCN infused approximately 37 min after PAPP dosing. WR242511 and PAHP were administered orally, either in crystalline form (WR242511) or dissolved in PEG200 (PAHP), and NaCN infused once the MHb level had peaked and was about 5 percent. The TRA, infused NaCN and percent MHb means for the three compounds were not significantly different from each other. The presence of MHb in the blood, and its availability to tie up CN^-, appears to be the important factor in mitigating the toxic effects of NaCN, and the variations in dosing protocols and time delays prior to NaCN infusion for each compound did not appear to create differences in efficacy. To determine the dosage of PAHP required to produce approximately 5 percent MHb, six animals were dosed with gelatin capsules containing either 5.0 mg PAHP/kg BW (using approximately 120 mg/mL PAHP in PEG200) or the vehicle (PEG200). The pilot study is reported in Appendix J. Based on results and discussions with the COR, the PAHP dosage was increased to 7 mg/kg BW and animals were challenged with NaCN when MHb was in the 3 to 7 percent range. After a one-week PAHP washout following the pilot study, Phase VIII began on June 20,1994.

Phase V - Neither hydroxylamine nor other therapy was required in any animal pretreated with PAPP and infused with a 2x AvTRA NaCN dose. Several animals did have up to a 40 sec pause in respiration starting approximately 20 sec after the NaCN infusion ceased. This is believed to be due to physiologic compensatory responses to histotoxic hypoxia and metabolic acidosis. In CN^- poisoning, the initial effects result from a cell's diminished ability to utilize delivered oxygen. At the cellular level, CN^- inhibits cytochrome oxidase in the electron transport system, which is a part of the oxidative pathway for the breakdown of glucose to release energy, carbon dioxide, and water for cellular functions. Inhibition of cytochrome oxidase forces the cell to use an anaerobic pathway to produce energy and lactic acid. Continued NaCN infusion increases the lactic acid concentration, which diffuses out of the cell and into the blood plasma, resulting in a metabolic acidosis. Compensatory mechanisms for acid-base regulation in an organism include respiratory control and renal mechanisms. According to Mountcastle's Medical Physiology, [17] the introduction of a nonvolatile fixed acid, such as lactic acid, causes an immediate decrease in plasma pH, an increase in carbonic acid content, and a rise in pCO_2. The rise in pCO_2 and increase in H^+

concentration stimulates respiration, resulting in tachypnea and hyperventilation. Ventilation remains above normal as long as the H^+ concentration remains elevated. When NaCN infusion is stopped, lactic acid production slows, and carbon dioxide concentration in blood is reduced; the respiratory compensatory mechanism ceases, which may result in a brief temporary pause in respiration. The respiratory pause continues until the pCO_2 returns to normal. In the Study Director's opinion, a respiratory pause and not respiratory arrest was observed in these dogs.

6.0 RECORD ARCHIVES

Records pertaining to the conduct of this study are contained in Battelle laboratory record books which are specific for this task. These records and the final report will be archived at Battelle or other archive facility designated by the U.S. Army. NaCN dosing solutions were infused and samples provided for concentration analyses. Excess NaCN dosing solutions have been destroyed. Samples of test MHb-forming compounds, crystalline NaCN, hydroxylamine, atropine sulfate, methylene blue, and sodium thiosulfate, will be maintained at the MREF for 5 yr or until returned to the U.S. Army. Blood samples have been destroyed.

7.0 ACKNOWLEDGMENTS

The names, titles, and degrees or certification of the principal contributors to this study are listed below:

Name	Title	Degree
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APPENDIX A

MREF PROTOCOL 98 INCLUDING AMENDMENTS, ADDENDA AND STUDY DEVIATIONS

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Study Performed by Battelle Memorial Institute
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9. Introduction:

Current therapy for cyanide (CN) intoxication consists of the intravenous (I.V.) administration of sodium nitrite and sodium thiosulfate. Field use of this treatment regimen is impractical for three reasons:

1) To be effective in counteracting CN intoxication, life saving therapy must be administered very rapidly,

2) Sodium nitrite therapy must be administered I.V., and

3) The potential for hypotensive effects resulting from sodium nitrite administration requires that treatment be performed under medical supervision.

A preventative or prophylactic treatment regimen for CN intoxication, therefore, needs to be established to eliminate the field use constraints of current therapy. One approach is to pretreat with a drug that induces methemoglobin (MHb) formation to remove the CN ion from the cellular respiratory enzyme, cytochrome oxidase. While data exist to support the success of pretreatment therapy in various animal models, there has not

been a study in which different pretreatment regimens were evaluated concurrently in the preferred canine animal model.

Historically, efficacy evaluations of anticyanide therapeutic compounds used lethality as the toxic endpoint in unanesthetized animals. In 1991, Vick and Froehlich developed an anesthetized canine model which they administered an intravenous (I.V.) CN bolus and used lethality as a measure of the efficacy of MHb-forming therapeutic regimens.[1] This model has been modified to utilize a slow I.V. infusion of CN leading to respiratory arrest.[2] This modification of the Vick-Froehlich canine model replaces the classical use of lethality (LD_{50}) as the endpoint for toxicity assessment with respiratory arrest for 30 seconds, and has the advantage of permitting multiple evaluations of MHb-forming therapeutic regimens in the same animal. Other animal models currently are not available for such evaluations because of differences in enzymatic profiles and kinetics of MHb formation between man and other species.[3,8,9,10,11,12]

10. Objectives:

In this study, two MHb-forming compounds are evaluated for efficacy in countering the toxic effects of sodium cyanide (NaCN). The principal objectives are to:

1) determine the protective efficacy provided by low levels (less than or equal to 10 percent) of methemoglobinemia against NaCN exposure, and

2) compare and quantitate the efficacy of long-acting and short-acting MHb-forming compounds in protecting against NaCN exposure.

This study is conducted at Battelle's MREF under the Good Laboratory Practice (GLP) guidelines of the Food and Drug Administration (FDA).

11. Experimental Design:

A. Test System

(1) Animals - Male Beagle dogs, Canis familiaris, have been specified for use in this study. The dog is the preferred animal model for anti-CN, MHb-inducing drug testing as the enzymatic profiles and kinetics of MHb formation are similar for dog and man.[3] There is considerable scientific evidence that canine MHb reductase activity is similar to that of man and, therefore, predictive of MHb responses in man.[4,5,6,7] The MHb reductase activity in rodents is significantly greater than that of man.[8,9] Felines do not conjugate phenols to glucuronic acid

(lack glucuronyl transferase), resulting in the inability to metabolize many of the MHb-forming drugs.[10] Swine have been recommended as an appropriate model; however, their primary energy source for MHb reduction is lactate as opposed to glucose in dogs and humans.[11] Since CN intoxication produces a severe lactate acidosis[12], further development of an *in vivo* swine model will depend upon the impact of the differing energy source on MHb reduction in the two species. Dogs have been historically used as the animal model of choice for studying CN therapy and an extensive data base exists.[3] The USAMRDC has selected the dog for use in their Decision Tree Network for CN intoxication treatment studies. Additionally, this species is recommended by the North Atlantic Treaty Organization scientists in the December 1991 findings of the Research Study Group-3 panel for CN research. The extensive canine data base also contains information on MHb formation/reduction kinetics for several 8-aminoquinolines proposed as MHb-forming, CN-treatment regimens.

This study is conducted in five phases to minimize the number of animals needed to achieve statistically valid results. Animals are anesthetized during NaCN infusion to minimize any discomfort, injury, or anxiety. Animals are closely monitored throughout the study for signs (anorexia, severe dehydration, excessive weight loss, lethargy, prostration) of discomfort. Should abnormal observations (food or water intake, weight loss, behavior, etc.) be reported, appropriate care will be provided by a MREF and/or Study Veterinarian. If, in the opinion of the Study Veterinarian or the Study Director, a dog appears to be suffering or in a moribund state, that animal will be euthanized with an overdose of sodium pentobarbital, approved euthanasia solution.

Protocols of all experiments using animals are reviewed and approved by Battelle's Institutional Animal Care and Use Committee (IACUC) prior to initiation of the study. The proper care and use of animals in the conduct of research described in this protocol is the responsibility of the Study Veterinarian, the Study Director, and MREF Management.

(2) Initial Weight - Dogs placed on study weigh a minimum of 9 kg.

(3) Quarantine - Dogs received at Battelle undergo a minimum 14 days of quarantine as specified in Battelle Standard Operating Procedure (SOP) ARF II-019, "Standard Operating Procedure for Test System Quarantine". All animals are examined by the Study Veterinarian or his designee within the quarantine period. Blood samples are taken for hematology and serum chemistry

evaluation. Fecal samples are taken for evaluation of gastrointestinal parasite infestation.

(4) Animal Identification - Animals are received with tattoos. If a dog arrives without a tattoo or with an identification number that duplicates another animal's number, a new tattoo is applied in accordance with Battelle SOP ARF II-009, "Standard Operating Procedure for Test System Identification".

(5) Housing - Dogs are housed in stainless-steel cages that comply with the 1991 revised Animal Welfare Act and allow individual housing during the recovery of the animal and as specified in Battelle SOP ARF II-012, "Standard Operating Procedure for Cage and Rack Specification".

(6) Lighting - Fluorescent lighting is used with a light/dark cycle of 12 hours each per day.

(7) Temperature - The air temperature in rooms in which dogs are housed is maintained at 65-84 degrees F. At least 95 percent of the total recordings will fall within the specified range.

(8) Humidity - Relative humidity in any room in which dogs are housed is maintained at 30-70 percent. At least 95 percent of the total recordings will fall within the specified range.

(9) Diet - Purina (St. Louis, MO) certified dog chow is fed daily in accordance with Battelle SOP ARF II-017, "Standard Operating Procedure for Feeding". No contaminants that would interfere with the results of the study are known to be present in the feed. Analyses of the feed can be obtained from Purina. Animals are fasted prior to anesthetization, and fed following the experiment when they are alert and fully recovered from anesthetic effects.

(10) Water - Water is supplied from the Battelle water system and given ad libitum through a water bowl watering system. No contaminants that would interfere with the results of the study are known to be present in the water. Water is analyzed annually for potability and for contaminants.

(11) Animal Selection - Based on physical examinations and clinical laboratory findings, acceptable animals are identified by the Study Director and Study Veterinarian or his designee.

(12) Study Preparation - Assignment to study phases and dosages of pretreatments are based upon individual animal body weight. All dogs are weighed within 24 hours of each experiment. The area

over cephalic, saphenous and jugular veins are clipped of hair for catheter placement. Vessels chosen for drug infusion and blood withdrawal will be as consistent as possible for each animal within the study and identified on the experimental worksheet. Areas for cardiac electrode attachment are clipped of hair.

Anesthesia is instituted using sodium pentobarbital to effect (generally 25-32 mg/kg body weight). Anesthetized dogs are intubated and catheter sites prepped. Each animal is positioned to allow for instrumentation and the animal allowed to equilibrate prior to taking baseline data. An attempt is made to make the preparation and equilibration times consistent for all animals. Baseline data are collected for a minimum of five minutes. Physiologic data recorded includes air flow, respiratory rate, and heart rate, using lead II of the electrocardiogram (ECG). Instrumentation of animals is described in Battelle SOP MREF VI-010.

(13) Battelle's Animal Resources Facilities have been registered with the U.S. Department of Agriculture (USDA) as a Research Facility (Number 31-R-021) since August 14, 1967, and are periodically inspected in accordance with the provisions of the Federal Animal Welfare Act. Battelle's statement of assurance regarding the Department of Health and Human Services (DHHS) policy on humane care of laboratory animals was accepted by the Office of Protection from Research Risks, National Institutes of Health (NIH) on August 27, 1973. Animals at Battelle are cared for in accordance with the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 86-23) and/or in the regulations and standards as promulgated by the Agricultural Research Service, USDA, pursuant to the Laboratory Animal Welfare Act of August 24, 1966, as amended.

(14) On January 31, 1978, Battelle received full accreditation of its animal care programs and facilities from the American Association for Accreditation of Laboratory Animal Care (AAALAC). Battelle's full accreditation status has been renewed after every inspection since the original accreditation. The MREF is a part of the facilities granted full accreditation.

B. Test Material

(1) Treatment Compounds - Two pretreatment drugs (one short-acting MHb former and the other long-acting) and hydroxylamine are provided by USAMRDC. The identity and purity analyses of these compounds are provided by USAMRDC and will not be duplicated by

Battelle. Concentrations of pretreatment dosing solutions are confirmed by Battelle chemists.

(2) Toxic Agent - NaCN is purchased from a commercial source. Purity, appropriate identification (batch number, lot number, state), and stability data is provided by the supplier. A saline dosing solution of 4 mg NaCN/mL is prepared daily prior to the initiation of that day's experiment(s). The concentration of CN in the dosing solution is confirmed by Battelle chemists.

(3) Safety procedures for the use of chemical agents are thoroughly outlined in facility plans, in personnel requirements for qualification to work with test compounds and NaCN, and in standard operating procedures for storage and use of test compounds. Refer to Battelle SOP MREF VII-011 entitled "Standard Operating Procedure for Receipt, Inventory, Storage, Handling, Packaging, Shipping, and Disposal of Experimental Test Articles at the MREF" and Battelle SOP MREF IX-001 entitled "Standard Operating Procedure for Use of Cyanide Salts/Solutions".

C. Study Design

Phases I and II studies are conducted concurrently to reduce the time necessary to complete the study. Phase II may commence before Phase I. Within 30 working days of the completion of each phase, an letter report and unanalyzed data summary are submitted to the U.S. Army Contracting Officer's Representative (COR) and technical Point of Contact (POC) for evaluation. The five phases are summarized in the table below.

EXPERIMENTAL DESIGN SUMMARY

Phase	Description of Experiments	n
I	Repeated NaCN Dosing To Effect	4
II	Limited Methemoglobin Kinetic Studies with Two Compounds	4/compound Total 8
III	Efficacy Experiments for Four Doses of a Short-Acting Methemoglobin Former	8[a]
IV	Comparative Efficacy Experiments for Two Methemoglobinemia Formers	9[a]
V	Fixed NaCN Challenge of Dogs Given a Methemoglobin Forming Compound	10[b]

[a] Some of these animals may be dogs used in Phases I and II.
[b] These animals will include dogs used in Phases III and IV.

All phases, except II, require blood collection for CN, total Hb and MHb analysis. Phase II requires only Hb and MHb analyses. Blood is drawn, heparinized and placed on ice or immediately analyzed for Hb and MHb. Hemoglobin and MHb levels are analyzed using an automated co-oximeter. Blood CN (total and/or plasma) is analyzed using the Technicon analyzer in accordance with a SOP provided by the Army (method of Groff et al[13]). Times for blood collection will be determined in consultation with the COR and POC. If total blood volume drawn on days of experimentation is necessarily large such that an anemic state results, the amount of time between experiments is increased to allow dogs to return to a normal physiologic state.

Multiple experimentation in the same animal may give rise to carryover effects from one experiment to the next. Carryover effects may be due to repeated use of an anesthetic, repeated NaCN exposure plus hydroxylamine therapy, or pathologic/physiologic changes resulting from respiratory arrest. If carryover effects are observed during the course of the study, consultation with the COR and the technical POC is initiated to determine if changes to the study are needed. The first phase addresses the feasibility of multiple experimentation in the same anesthetized animal.

(1) PHASE I - EFFECTS OF REPEATED INFUSION PLUS THERAPY IN ANESTHETIZED DOGS

Phase I of the study is designed to determine the variability in time to respiratory arrest (the NaCN effect), survivability of anesthetized dogs repeatedly infused with NaCN and given I.V. hydroxylamine (10 mg/kg) therapy, replicability of the procedure, and development of trends in the data. In Phase I, four dogs are anesthetized, catheterized, intubated and instrumented to record air flow, respiratory rate, and ECG. Immediately following baseline data collection, a 4 mg NaCN/mL saline solution is infused at a rate of 2.0 mL/min. Infusion ceases when respiratory arrest occurs. Thirty seconds following respiratory arrest, the animal is revived with 10 mg hydroxylamine/kg given I.V. and artificial respiration as needed. Additional therapies, such as methylene blue and atropine sulfate injection may be instituted to ensure the survival of the animals. Parameters to be measured and recorded include: blood CN (total and/or plasma) levels, Hb, percent MHb, rate of respiration, air flow, and heart rate.

Each of four animals is exposed to this experimental regimen four times, with a minimum of one week between exposures. Animals from this phase may be used in succeeding phases, depending on animal health status and washout times of compounds and drugs administered. Data summary and a letter report from

Phase I experiments are presented to the COR and technical POC to assess the feasibility of multiple experiments in the same anesthetized canine. If lethality occurs, tolerance develops to the anesthetic agent, or large changes in the time to respiratory arrest for an animal over the four experimental procedures, it may become necessary to modify Phases III, IV and V. Should the results from these experiments indicate problems with the anesthetic (sodium pentobarbital) and/or method of administration (I.V.), then use of an inhalation anesthetic may be examined in a few animals.

(2) PHASE II - LIMITED KINETIC EXPERIMENTS

Phase II consists of limited MHb kinetic experiments for each test compound. The test compounds, plus information to estimate the washout time period and the four target doses (i.e., doses of each test compound required to produce A = 2.5%, B = 5%, C = 10%, and D = 15% methemoglobinemia) is provided by USAMRDC. The kinetic experiments are designed to establish a test compound dose-response curve for MHb formation over time in this population of animals. The experiment is a four period cross-over design, in which four dogs per test compound and four doses per compound are used as shown below.

PHASE II EXPERIMENTAL DESIGN[a]

Animal	Period 1	2	3	4
1	A	B	C	D
2	B	C	D	A
3	D	A	B	C
4	C	D	A	B

[a] Doses - A ≃ 2.5%, B ≃ 5%, C ≃ 10%, D ≃ 15% methemoglobinemia

Each period contains four doses of the test compound being evaluated and each dog receives each of the four doses with a washout period between each dose. All dogs used in this phase are catheterized for blood collection but are not instrumented for cardiac and respiratory data collection. Animals used with the short-acting MHb-forming compound are anesthetized prior to administration of the pretreatment compound in order to account for effects on metabolism of the pretreatment compound due to the anesthetic. The short-acting, MHb-forming compound is

formulated for I.V. administration per instructions from the COR, and injected as a bolus. Blood is collected for Hb and MHb analysis only. Times blood is to be collected will be determined in consultation with the COR and POC.

The long-acting compound is administered per os in a gelatin capsule. If vomiting occurs within 30 min, the drug is re-administered; vomiting after 30 min may preclude further sampling (at the discretion of the Study Director). The blood collection times for the longer-acting MHb-former are provided by the COR or determined from a preliminary study in a few dogs.

Dose-response data for MHb formation over time for each test compound and each assists in estimating the following parameters to be used in Phases III, IV and V:

1) The variation among animals,

2) The test compound doses required to induce a methemoglobinemia between 2.5 and 15 percent [maximum MHb concentration (C_{max})],

3) The pretreatment dosing times [length of time (t_{max}) to reach C_{max}],

4) The blood sampling intervals, and

5) Washout time periods (elimination half-life for each test compound).

Kinetic experiments are conducted with both test compounds. Phase III testing of the short-acting, MHb-forming compound may begin following satisfactory completion of its MHb kinetic analyses in Phase II even though kinetic evaluation of the long-acting MHb-forming compound has not been completed.

(3) PHASE III - EFFICACY EXPERIMENTS FOR FOUR DOSES OF A SHORT-ACTING METHEMOGLOBIN-FORMING COMPOUND

Phase III is designed to determine the efficacy of a short-acting, MHb-forming compound, at doses expected to induce a methemoglobinemia of approximately 0, 2.5, 5, and 10 percent, in delaying CN-induced respiratory arrest. The experimental design is a four period, cross-over design as shown below, using a total of eight animals in a randomized block.

PHASE III TEST PLAN[a]

Animal	Period			
	1	2	3	4
1	A	B	D	C
2	B	C	A	D
3	C	D	B	A
4	D	A	C	B
5	A	B	D	C
6	B	C	A	D
7	C	D	B	A
8	D	A	C	B

[a] Dose - A ≃ 0%, B ≃ 2.5%, C ≃ 5%, D ≃ 10% methemoglobinemia

Kinetic information derived in Phase II is used to select target doses (i.e, A = 0%, B = 2.5%, C = 5%, D = 10% methemoglobinemia) for the pretreatment test compound, as well as pretreatment dosing time prior to NaCN challenge, and blood sampling intervals. If data generated in Phase II indicate that the average MHb level at the lowest test compound dose (target 2.5 percent MHb level) is not statistically greater than the baseline normal MHb level, then alternative test compound doses predicted to produce MHb levels between 2.5 and 15 percent are selected. Each period of Phase III testing contains all four target doses and each animal receives each dose with a washout period between dosings. The short-acting, MHb-forming test compound is administered as previously described in Phase II. Dogs are anesthetized, intubated, catheterized, instrumented, infused with NaCN, administered hydroxylamine therapy, and data recorded. NaCN infusion is initiated at the time the predicted MHb level is reached based on results obtained in Phase II. Blood samples are collected at times determined from Phase II data to monitor blood CN (total and/or plasma) levels and MHb levels.

(4) PHASE IV - COMPARISON OF TWO METHEMOGLOBIN-FORMING COMPOUNDS AT A SIMILAR METHEMOGLOBIN LEVEL (5 PERCENT)

Phase IV is designed to compare the efficacy of two MHb-forming compounds, at a similar MHb level, in delaying CN-induced respiratory arrest. Phase IV is revised if analysis of data in Phases II and III indicates effects on metabolism of compounds due to anesthetic administration. The experiment is a three-period design using nine randomly numbered animals as shown below. If there is an indication that there is a difference in compound treatment efficacy, then additional animals (up to 6) may be needed to show statistically significant differences. Test compounds are identified by the subscripts "s" and "l" for the short-acting and long-acting, MHb-forming compounds, respectively. A dose targeting 5 percent methemoglobinemia and time of administration for the short-acting, MHb-forming compound are determined from data from Phases II and III. The target MHb level at the time of NaCN injection for the long-acting, MHb-forming compound is 5 percent. Each animal serves as its own control.

PHASE IV EXPERIMENTAL DESIGN[a]

Animal	Period		
	1	2	3
1	A	B_s	B_l
2	A	B_s	B_l
3	A	B_s	B_l
4	A	B_s	B_l
5	A	B_s	B_l
6	A	B_s	B_l
7	A	B_s	B_l
8	A	B_s	B_l
9	A	B_s	B_l

[a] Doses - A ≃ 0, B ≃ 5% methemoglobinemia
$_l$ Long-acting, MHb-forming compound
$_s$ Short-acting, MHb-forming compound

Dogs are anesthetized, intubated, catheterized, and instrumented, and data recorded as described in Section 11.A.(12) and Phase I. Following baseline data collection, animals are infused with NaCN, until respiratory arrest. Thirty seconds after respiratory arrest, they receive hydroxylamine therapy. While it would be ideal to randomize use of the short-acting and long-acting, MHb-forming compounds over the second and third periods, the long elimination half-life of the long-acting, MHb-forming compound precludes this. Therefore, the two MHb-forming compounds are examined in a systematic fashion. In the second period, animals are pretreated with a dose of the short-acting, MHb-forming compound predicted to produce 5 percent MHb. In the third period, each animal is administered the long-acting, MHb-forming compound at the dose expected to produce 5 percent MHb, a level comparable to that achieved with the short-acting compound. Dose, dosing time, washout time period, and sampling intervals are selected for each compound based on results from previous Phases.

(5) PHASE V - EFFICACY OF MHb-INDUCING DRUGS IN COUNTERING THE EFFECTS OF NaCN ADMINISTERED AT TWICE THE DOSE CAUSING RESPIRATORY ARREST OF UNTREATED ANIMALS

This final phase is designed to determine the pretreatment efficacy of a MHb-forming compound in countering the lethal effects of NaCN administered at 2X the average dose resulting in respiratory arrest. The average dose resulting in respiratory arrest is determined by taking the average of the doses calculated from the average time to respiratory arrest (AvTRA) of untreated animals and multiplying this by the fixed infusion rate and concentration of the NaCN saline solution. The AvTRA is determined from the data of Phases I, III, and IV. If the variability of the data is large, the 2X AvTRA may be determined for each individual animal used in the study. Predicted therapeutic doses of the MHb formers are used to determine efficacy. Dose, dosing time following administration of the MHb-forming compound, and blood sampling intervals are selected based on results from previous phases. Dogs are anesthetized, intubated, catheterized, instrumented, infused with NaCN, and data recorded as described in Section 11.A.(12) and Phase I. Hydroxylamine treatment is administered as previously described. Artificial respiration and additional therapies, as needed, are given to ensure survival. Animals used in Phases III and IV, following a washout period, may be used in this phase.

D. Administration of Sodium Cyanide, Pretreatments and Treatments

The short-acting MHb-forming compound is administered to the animals by the I.V. route in order to minimize data variability. The long-acting MHb-forming compound is administered orally in gelatin capsules as requested by USAMRDC. NaCN is administered I.V. on a mg/mL/min dosage basis using an infusion pump. Hydroxylamine (10 mg/kg) is given I.V. through the infusion catheter.

E. Measurements Recorded

Blood cyanide (total and/or plasma) - The total CN can be analyzed at a later time after the CN has been stabilized in the sample. Plasma CN should be analyzed as soon as possible.
Body weights - Prior to anesthesia
Air Flow - tidal volume and/or minute volume
Respiratory rate
Heart rate using the ECG
Total hemoglobin
Percent MHb - Performed by automated co-oximeter (OSM^{TM}3 Radiometer in the dog setting)
Dose of NaCN required to cause cessation of respiration for 30 sec
Time to respiratory arrest - Start of NaCN infusion to cessation of spontaneous breathing as measured by respiratory rate and air flow.

Respiratory and cardiovascular events are recorded using a physiological monitoring, computerized data acquisition, analysis and archiving system.

F. Disposition of Experimental Animals

The Study Veterinarian and Study Director are responsible for assessing the condition of animals on study. If conditions exist such that continuation on study would be inhumane, the animal is euthanized. The determination for euthanasia is based on the criteria presented in Battelle SOP ARF II-003. Animals are euthanized in accordance with Battelle SOP ARF II-037. Animals that die on study, or are sacrificed in a moribund condition, are cremated. No pathology is performed. Surviving animals are disposed of humanely, either by reassignment or euthanasia.

12. Statistical Approach:

Average values of blood CN (total and/or plasma) levels, total hemoglobin, percent MHb, rate of respiration, air flow, heart rate, and time to respiratory arrest from Phase I experiments are plotted against

experimental sequence number to assess the effect of multiple experiments in the same anesthetized canine. Blood CN (total and/or plasma) levels, total hemoglobin, and percent MHb coded for experimental sequence are plotted against time for each animal. In addition, an analysis of variance is carried out on the time to respiratory arrest data to determine the effects of repeated dosing.

MHb C_{max}, t_{max}, and elimination half-lives are empirically estimated from the percent MHb versus time curves for each animal and test compound dose tested in Phase II. Analyses of variance are carried out to assess the effects of test compound dose and to estimate the extent of variation between animals for each of these kinetic parameters. If C_{max} is statistically related to test compound dose, then a linear regression model is fitted to the C_{max} data to estimate the test compound doses required to induce a methemoglobinemia between 2.5 and 15 percent.

An analysis of variance appropriate for crossover experiments is performed with the data collected in Phase III to determine the effects of MHb level on time to respiratory arrest. The mean protective ratio (average time to respiratory arrest with animals having received pretreatment compound divided by average time to respiratory arrest with animals not having received pretreatment compound) is estimated for each level of methemoglobinemia induced by the short-acting drug. Blood CN (total and/or plasma) levels, total hemoglobin, percent MHb, rate of respiration, air flow, heart rate, and time to respiratory arrest from Phase III experiments are plotted against MHb level and time.

The difference between the time to respiratory arrest observed for the short-acting and long-acting MHb-forming compounds is calculated for each animal in Phase IV. An analysis of variance is computed on the differences to determine if there are any statistical differences in the protection provided by these two drugs at doses producing similar MHb levels.

In Phase V experiments, ten dogs are administered a therapeutic dose of a MHb-former and then dosed at 2 times the AvTRA. Ninety-five percent confidence limits are computed for the incidence of respiratory arrest for specified time intervals. Ninety-five percent confidence limits may also be computed for the mean time to respiratory arrest for this group of animals.

13. Records to be Maintained:

A. Preparation of reagents, dose analyses and dosage administration

B. Animal data

C. Recorded measurements

D. Mortality data

E. NaCN decontamination, monitoring, and disposal records.

14. Reports:

A. A draft final report is prepared within 30 work days after completion of exposures and analyses of the data. The draft final report includes:

1) Signature page of key personnel
2) Experimental design
3) Animal selection criteria
4) Test material description, analyses, preparation, and administration
5) Recorded measurements
6) Statistical analyses of data
7) Discussions and conclusions

B. Following receipt of draft final report comments from USAMRDC, a final report is prepared within 30 work days.

C. Following completion of the study, a minimum of one collaborative article (with USAMRDC) will be prepared and submitted to a refereed journal.

15. References:

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16. Approval Signatures:

David W. Hobson
David W. Hobson, Ph.D.
MREF Principal Investigator and Manager
16 Jul 93
Date

Frances M. Reid
Frances M. Reid, D.V.M., M.S.
Study Director
7-14-93
Date

David L. Stitcher
David L. Stitcher, CIH
Safety and Surety Officer
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Date

Ronald Menton
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7/23/93
Date

Richard A. Sharp
Quality Assurance Unit
7/30/93
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Allan G. Manus
A. G. Manus, D.V.M., M.Sc.
Study Veterinarian
7/23/93
Date

Don W. Korte, Jr. LTC, MS
LTC Don W. Korte, Jr., Ph.D.
USAMRICD COR
2 Aug 93
Date

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Amendment No. 1

Change: Add the following sentence to Section 11.A.(1)

"Beagles, with a minimum body weight of 9 kg and a minimum age of 6 months, are purchased from Hazleton Laboratories."

Reason: At the time the protocol was written, the source and age range had not been specified. Now that the source has been identified and the age of the animals determined for the desired minimum weight, this information is being incorporated into the protocol.

Impact on the Study: There are no adverse affects on the study. A more detailed description of the test system is given.

Change: Delete the first sentence of the second paragraph in Section 11.A.(12), "Study Preparation", which reads:

"Anesthesia is instituted using sodium pentobarbital to effect (generally 25-32 mg/kg body weight)."

substitute with the following sentence:

"Anesthesia is instituted using sodium pentobarbital (generally 25-32 mg/kg body weight) or a combination of thiamylal (approximately 5 mg/kg) and sodium pentobarbital to effect."

Reason: The combination regimen maintains an anesthetic effect for a shorter time period, but sufficient to perform the necessary procedure. The time to full recovery is reduced. Complete recovery from sodium pentobarbital anesthesia requires approximately 8-24 hours. With the anesthetic combination, in our experience, complete recovery requires approximately 8 hours. The shorter recovery time and the reduction in amount of sodium pentobarbital necessary to reach the desired plan of anesthesia makes the combination anesthetic regimen more desirable.

Impact on the Study: This change should not adversely affect results of the study. No adverse health effects or changes in experimental parameters have been observed as a result

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of using this regimen. Animals used for training personnel on procedures to be used in Task 92-28 and for validation of equipment were anesthetized using either of the anesthetic regimens discussed above. A limited comparison of the data did not reveal any significant changes in heart rate or respiratory rate between the two anesthetics.

Change: Delete the second sentence of Section 11.B.(1), "Treatment Compounds" which reads

"The identity and purity analyses of these compounds are provided by USAMRDC and will not be duplicated by Battelle."

and replace it with the following:

"The identity, composition, stability, and purity analyses of these compounds are the responsibility of USAMRDC and will not be duplicated by Battelle."

Reason: Good Laboratory Practice (GLP) Regulations, 21 CFR Part 58, Part VI, Subpart F, §58.105 (a); states that "The identity, strength, purity and composition or other characteristics which appropriately define the test or control article shall be determined for each batch and shall be documented." The regulations require the above information to be documented and a part of the study file. The reference to composition and stability analyses was inadvertently deleted from the original protocol.

Impact on the Study: There are no adverse affects on the study with the addition of this information. The addition of "composition and stability" information for compounds to be provided by the sponsor addresses the GLP requirements for test and control articles. Information provided by the sponsor will be placed in the study files.

Change: In Section 11.C.(2), "Phase II - Limited Kinetic Experiments", delete the second sentence of the second paragraph which reads:

"All dogs used in this phase are catheterized for blood collection but are not instrumented for cardiac and respiratory data collection."

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Substitute with the following sentence:

"Dogs used in this phase may be catheterized for blood collection but are not instrumented for cardiac and respiratory data collection."

Reason: Previous studies indicate that after dosing the long-acting methemoglobin-forming compound (WR242511AE), 2-3 days are necessary for methemoglobin levels to reach a peak and that approximately 30 days are required for the methemoglobin level to return to normal. Catheterization is not deemed necessary to draw the small (approximately 0.3 mL) amount of blood necessary for analysis of total hemoglobin and methemoglobin over this prolonged time period. Blood sample collection using an appropriate syringe and needle is adequate and would be less stressful on the animals.

Impact of Change: The proposed change does not have an adverse effect on the study. The proposed technique is expected to minimize handling and stress on the dogs.

Approval Signatures for Amendment No. 1:

Frances M. Reid — 9-28-93
Frances M. Reid, D.V.M., M.S. — Date
Study Director

David L. Stitcher — 9-28-93
David L. Stitcher, CIH — Date
Safety and Surety Officer

Don W. Korte, Jr. — 28 Sep 93
LTC Don W. Korte, Jr., Ph.D. — Date
USAMRICD COR

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ADDENDUM NO. 1 TO MREF PROTOCOL 98

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Add Phase VI

Introduction:

Compounds that produce methemoglobin (MHb) are potentially useful as prophylactic anticyanide compounds. Data exist to support such pretreatment therapy in various animal models. The extent and duration of the MHb formation are very important when considering the MHb-former as a prophylactic treatment regimen. For example, a compound that produces a high MHb level within minutes and lasts less than an hour or so would not be desirable. A compound that produces a moderate level of MHb within several hours and lasts 24 hours would be ideal. Task 92-28 evaluates the efficacy of two pretreatment MHb-forming compounds administered as a single dose to an anesthetized canine model. However, the task, does not address the effect of multiple dosing on maintaining a specified level of MHb in the blood for a specified period of time, nor does it establish a dosing interval for the two compounds. Phase VI is being added to perform a comparison multiple-dose study to test the ability of two candidate compounds to produce sufficient but nontoxic levels of MHb over a period of time greater than two hours.

Objectives:

In this phase, two MHb-forming compounds are evaluated in a limited pharmacodynamic and pharmacokinetic study with multiple doses of compounds WR242511 and WR269410 (PAHP). The principal objectives are to:

1) evaluate the probability of either compound being able to produce an essentially constant level ("steady state") of methemoglobin.

2) determine, for each compound, if the dosing regimen evaluated is sufficient to produce "steady state" levels of methemoglobin, and

3) determine the maximum and minimum levels of MHb immediately prior to and at specified times after dosing of each compound.

This study is conducted at Battelle's Medical Research and Evaluation Facility under the Good Laboratory Practice (GLP) guidelines of the Food and Drug Administration (FDA).

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Experimental Design - Phase VI:

A. Test System

The male beagle dogs used for Phase VI are animals which have been used in earlier phases of Task 92-28. Animals to be dosed with compound WR269410 will need to be acclimated to a sling prior to dosing.

B. Test Material

Two MHb-forming compounds are to be provided by USAMRDC. The compounds are WR242511 and WR269410. The identity, composition, purity and stability analyses of these compounds are the responsibility of USAMRDC and will not be duplicated by Battelle.

Compound WR242511 will be administered orally via capsules. The weights of dogs as determined within 48 hours of study initiation will be used to determine the amounts of the compound to be weighed and placed into capsules for each dosing. All capsules to be administered during Phase VI will be prepared in advance for each dog. Capsules will be stored in sealable, clean, appropriately-labeled vials. The loading dose capsule for each animal will be stored in a separate vial from the maintenance dose vials. Compound WR269410 will be dosed by oral gavage. A stock solution will be made and the concentration tested periodically during the experiment. The dose to be administered will be calculated based upon the weight of each dog as determined within 48 hr of study initiation.

C. Study Design

Phase VI consists of limited pharmacodynamic experiments for WR242511 and WR269410 (PAHP), MHb-forming compounds. The test compounds, initial doses, and dosing regimen will be provided by USAMRDC. A pilot experiment, using 2 animals per compound, will be conducted to estimate maximum and minimum blood MHb levels, effective dosing intervals, and dose-MHb effect. If adjustments of the dose, dosing interval, or blood sampling times are not required, then additional animals (up to four) per compound will be dosed under the same regimen. If adjustments to the dose, dosing interval, or blood sampling times are required to obtain desired MHb levels, it may be necessary to repeat the pilot study before dosing additional animals. The pilot study would be repeated in the same animals using a sufficient wash out period.

Heparinized blood samples of approximately 2 mL will be collected for MHb and test compound analyses. The MHb will be analyzed on the Radiometer Hemoximeter™ as soon as possible after sample collection.

The remainder of the sample will be centrifuged for plasma collection. The plasma will be stored at approximately -70 degrees C until the end of the study and then shipped to Walter Reed Army Institute of Research for compound analyses.

WR242511 - Compound WR242511 will be administered orally at a loading dose of 2 mg/kg on Day 0, and followed by a 1 mg/kg maintenance dose every 48 hours for at least 2 weeks. An example of a possible bleeding schedule for the pilot study is as follows:

> Baseline, 4, 6, 8, 12, and 24 hours, and then every 12 hours for up to 15 days. Additional blood samples, for MHb determination only, may be collected during the first week of dosing to determine times of approximate maximum and minimum MHb.

WR269410 - Compound WR269410 will be administered at 3 mg/kg by gavage every 8 hours for a 7-day period. An example of a possible bleeding schedule for the pilot study is as follows:

> Baseline, 15, 30, 60, 90, 120 minutes, and then 4, 6, and 8 hours after the first dose. Blood samples will be taken within 1 minute of subsequent dosing and at least 60 minutes after dosing for the remainder of the experiment. For the pilot experiment, additional blood samples for MHb determination only are recommended. Sampling for MHb concentration only will begin approximately 2 hours prior to the 8 hour maintenance dosing and every 30 minutes for 2 hours after maintenance dosing for the first 48 hours.

Additional animals may need to be identified to complete the 6 animal per compound dosing paradigm. Adjustments to the schedules, doses or dosing interval will be made only after consultation with the U.S. Army Contracting Officer's Representative and the technical Point of Contact.

Should vomiting occur within 30 minutes of dosing, then the complete prescribed dose of the drug is re-administered. Vomiting after 30 minutes may preclude further blood sampling. This will be at the discretion of the Study Director.

A 2-mL whole blood sample is collected at sampling times in Phase VI for the determination of MHb and plasma drug concentration. The data to be recorded are animal body weights, MHb and Hb values, dosing and sampling times, drug target dose, and calculated amount of drug administered. Plasma samples will be sent to WRAIR for analysis of compound concentration at the completion of all sample collections for this phase.

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Data Analysis:

MHb and Hb levels are plotted versus time. For both WR269410 and WR242511, MHb and Hb values at 1 min prior to dosing are used as baseline values. MHb and Hb level curves after dosing are smoothed to estimate time to steady state maximum levels of MHb. A table with MHb values measured for each animal will be supplied to WRAIR.

Analysis of the pharmacokinetic information for each drug is made after WRAIR has chemically analyzed the plasma and transferred data to Battelle. Drug data should be submitted to Battelle in a mutually acceptable format and time frame to assist completion of the report. Analyses described for the MHb and Hb data will be repeated for compound concentration data.

Reporting:

A letter report describing the work accomplished in Phase VI will be provided within 30 working days of completion of data sampling. If the MREF is to incorporate the drug analyses data, the letter report will be completed within 30 working days after receipt of pharmacokinetic data from WRAIR.

Approval Signatures for Addendum No. 1:

Frances M. Reid, D.V.M., M.S.
Study Director — Date: 2-17-94

David L. Stitcher, CIH
Safety and Surety Officer — Date: 2-18-94

LTC Don W. Korte, Jr., Ph.D.
USAMRICD COR — Date: 19MAY94

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Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Amendment No. 2

Change: Delete the first sentence of the second paragraph in Section II.A.(12), "Study Preparation", which was amended to read:

"Anesthesia is instituted using sodium pentobarbital (generally 25-32 mg/kg body weight) or a combination of thiamylal (approximately 5 mg/kg) and sodium pentobarbital to effect."

substitute with the following:

"Anesthesia is instituted using sodium pentobarbital (generally 25-32 mg/kg body weight) or a combination of a short-acting barbituate (e.g., thiamylal or pentothal, etc.) and sodium pentobarbital to effect."

Reasons for Changes:

During our recent procurement of the short-acting barbituate thiamylal, the manufacturer notified Battelle that thiamylal was no longer available because of problems with the precursor compound during manufacturing. The manufacturer and Study Veterinarian recommended pentothal as a replacement, since pentothal's properties, mode of action and results were similar to thiamylal. Both anesthetics exert their effect over approximately a 20-minute period and preparation of the animal exceeds this time frame, therefore, no interference from these compounds is expected. Pentothal has been procured and is used to complete the study. The short-acting barbituates are used to maintain a shorter recovery time and reduce the amount of sodium pentobarbital needed for anesthetic affect.

Impact on the Study:

This change will not affect the integrity of the study. Remaining available sources of thiamylal have been obtained by the Study Director, but supplies are insufficient to complete the study. Pentothal had to be used in place of thiamylal to complete Phase III. A limited comparison of the data did not reveal any significant changes in heart rate or respiratory rate between the two anesthetics. No adverse health effects or changes in experimental parameters have been observed.

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Approval Signatures for Amendment No. 2

Frances M. Reid, D.V.M., M.S.
Study Director

4-1-94
Date

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Safety and Surety Officer

4-1-94
Date

LTC Don W. Korte, Jr., Ph.D.
USAMRICD COR

14 APR 94
Date

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ADDENDUM NO. 2 TO MREF PROTOCOL 98

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Add Phases VII and VIII

Introduction:

Task 92-28 evaluates the efficacy of two pretreatment methemoglobin (MHb)-forming compounds (WR302AG and WR242511AE) at similar methemoglobin levels in an anesthetized canine model. The information collected in Task 92-28 will determine the compound or compounds that are transitioned for field-use development. Compound WR269410 (PAHP) is an additional methemoglobin-forming compound that the client would like evaluated in the efficacy experiments. Phase VII is a limited pharmacodynamic experiment for PAHP. Data from Phase VII will be used to determine a dose expected to produce the targeted 5 percent methemoglobinemia, peak MHb levels for various doses, time to peak MHb levels, and washout time in this population of dogs. Phase VII will mimic Phase II of Task 92-28. Phase VIII is added to determine the efficacy of PAHP at the targeted 5 percent methemoglobinemia, and this plan is designed to mimic Phase IV.

Objectives:

Phase VII is a limited pharmacodynamic study and is designed to estimate the washout time period and the 5 percent methemoglobinemia dose for PAHP. In Phase VIII, the principal objectives are to:

1) Determine the efficacy of PAHP in countering cyanide intoxication, and

2) Compute the protective efficacy of PAHP.

This study is conducted at Battelle's Medical Research and Evaluation Facility (MREF) under the Good Laboratory Practice (GLP) guidelines of the Food and Drug Administration (FDA).

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Experimental Design:

A. Test System

The male beagle dogs used in Phases VII and VIII are animals presently assigned to Task 92-28. Animals used for Phase VIII will be those used in Phase IV following a washout period of at least three weeks. MHb levels in Phase IV animals will be at baseline levels prior to initiating Phase VIII. If such restraint is required, animals will be acclimated to a sling prior to dosing.

B. Test Material

1. Test Material - PAHP will be provided by the U.S. Army Medical Research, Development, Acquisition and Logistics Command (USAMRDALC, Provisional). The identity, composition, purity, and stability for PAHP will be provided by USAMRDALC. Concentration of dosing solutions will be confirmed by Battelle chemists.

2. Toxic Agent - Sodium cyanide (CN) will be purchased from a commercial source. Purity, appropriate identification (batch number, lot number, state), and stability data will be provided by the supplier. A saline dosing solution of 4 mg sodium cyanide/mL will be prepared daily prior to the initiation of that day's experiment(s). The concentration of CN in the dosing solution will be confirmed by Battelle chemists.

3. Safety procedures for the use of chemical agents are thoroughly outlined in facility plans, in personnel requirements for qualification to work with test compounds and sodium cyanide, and in standard operating procedures for storage and use of test compounds.

C. Study Design

Phase VII: Phase VII is a limited pharmacodynamic study designed to estimate the dose of PAHP that produces approximately 5 percent methemoglobinemia. The experimental design plan is a three-period crossover design, as shown on the following page:

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6/7/94 KR

PHASE VII EXPERIMENTAL DESIGN

Animal	Period		
	1	2	3
1	A[a]	B[a]	C[a]
2	B[a]	C[a]	A[a]
3	C[a]	A[a]	B[a]
4	A[a]	C[a]	B[a]
5	B[a]	A[a]	C[a]
6	C[a]	B[a]	A[a]

[a] Doses - To be determined

Depending on the completeness of the data to be provided by the Army, a smaller two period cross-over design may be used to reduce the number of animal experiments.

Up to six dogs are dosed with one of a possible three doses of PAHP. The A, B, and C (possibly) doses are selected to produce approximately 5 percent methemoglobinemia, based on data provided by USAMRDALC. PAHP doses will be administered per os. Each animal will receive each dose, with a minimum one-week washout period between dosings. Experimental procedures and data collection will mimic as possible, those used in Phase II pharmacodynamic experiments.

Phase VIII: Phase VIII is designed to measure the efficacy of PAHP in delaying CN-induced respiratory arrest at MHb levels of approximating 5 percent. Animals used previously in Phase IV will be assigned to Phase VIII. The experimental design is a two-period cross-over design, as shown on the following page:

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6/7/94 KC

PHASE VIII EXPERIMENTAL DESIGN

	Period	
Animal	1	2
1	Vehicle Control	PAHP
2	PAHP	Vehicle Control
3	Vehicle Control	PAHP
4	PAHP	Vehicle Control
5	Vehicle Control	PAHP
6	PAHP	Vehicle Control
7	Vehicle Control	PAHP
8	PAHP	Vehicle Control
9	Vehicle Control	PAHP

Each of up to nine dogs is pretreated with PAHP and the vehicle control with a minimum one-week washout period between dosings. The dose of PAHP is selected to produce approximately 5 percent methemoglobinemia. The animals will be randomly assigned to dosing order. A minimum three-week period is required between Phases IV and VIII. The experiment will require approximately two weeks to complete.

Experimental procedures, data collection, and data evaluation are similar to those used in Phase IV. Methods utilized for vehicle control experiments will mimic those used in Phase IV. PAHP is administered similarly to those used in Phase VII. Blood sample times and data collection procedures are based on data obtained in Phase VII for PAHP.

Data Analysis:

Data analyses for PAHP in Phase VII will be the same as those used in Phase II. Similarly, Phase VIII data analysis procedures will be the same as those used in Phase IV.

Reporting:

Letter reports describing the results of Phases VII and VIII will be provided within 30 working days following completion of data analyses.

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Approval Signatures for Addendum No. 2:

Frances M. Reid — 5-4-94

Frances M. Reid, D.V.M., M.S.
Study Director — Date

David L. Stitcher — 5-5-94

David L. Stitcher, CIH
Safety and Surety Officer — Date

Don W. Korte LTC, MS — 19 MAY 94

LTC Don W. Korte, Jr., Ph.D.
USAMRICD COR — Date

[illegible] COPY
6/7/94

6/16/94 IA

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Amendment No. 3 refers to Addendum 1.

1. Change: Add the following sentence to the section Experimental Design - Phase VI: A. "Test System", after the first sentence of the paragraph.

   "Restraint of animals to be dosed may require the use of a sling or other appropriate methods as determined by the Study Director."

   Reasons for Change: When the protocol was originally written, compound dosing was expected to be by oral gavage. A pilot study indicated that compounds, particularly WR269410, could be administered by capsule and thus slings may not be needed. The Study Director can determine the appropriate method of restraint and the degree of restraint needed for dosing.

   Impact on the Study: This change will not affect the integrity of the study, but gives the Study Director flexibility to designate proper restraint methods suitable for the selected dosing technique.

2. Change: Delete the last sentence to the section Experimental Design - Phase VI: A. "Test System", which reads:

   "Animals to be dosed with compound WR269410 will need to be acclimated to a sling prior to dosing."

   Substitute with the following sentence:

   "If sling restraint is required, the animals will be acclimated to a sling prior to dosing."

   Reasons for Change: When the protocol was originally written, compound dosing was expected to be by oral gavage with the animals restrained by using slings. Since the compounds to be tested will be administered by capsule, slings may not be needed.

   Impact on the Study: This change will not affect the integrity of the study, but gives the Study Director flexibility to designate proper restraint methods suitable for the selected dosing technique.

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3. Change: Delete the sixth sentence of paragraph two in the section Experimental Design - Phase VI: B. "Test Material", which reads:

"Compound WR269410 will be dosed by oral gavage."

Substitute with the following sentence:

"Compound WR269410 may be dosed by capsule or oral gavage."

Reasons for Change: When the protocol was originally written, compound dosing was expected to be by oral gavage. A pilot study indicated that dosing of the compounds, particularly WR269410 in PEG 200, could be administered by capsule.

Impact on the Study: This change will not affect the integrity of the study. In previous phases, in which this compound has been similarly given, has been done using capsules. Dosing by capsule is believed to be the most favorable route of administration.

4. Change: Delete the remainder of the first paragraph after the second sentence in the section Experimental Design - Phase VI: C. "Study Design", which reads:

"A pilot experiment, using 2 animals per compound, will be conducted to estimate maximum and minimum blood MHb levels, effective dosing intervals, and dose-MHb effect. If adjustments of the dose, dosing interval, or blood sampling times are not required, then additional animals (up to four) per compound will be dosed under the same regimen. If adjustments to the dose, dosing interval, or blood sampling times are required to obtain desired MHb levels, it may be necessary to repeat the pilot study before dosing additional animals. The pilot study would be repeated in the same animals using a sufficient wash out period."

Substitute with the following:

"If sufficient information has been provided by Walter Reed Army Institute of Research (WRAIR), then the multiple-dosing experiment will be conducted using up to six animals per compound to estimate minimum and maximum blood MHb levels, effective dosing intervals, and dose-MHb effect. If insufficient information is available, then a pilot experiment, using 2 animals per compound, will be conducted to provide estimates of these parameters. If the pilot experiment is required, but adjustments of the dose, dosing interval, or blood sampling times are not required, then additional animals (up to

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four per compound) will be dosed under the same regimen. If adjustments to the dose, dosing interval, or blood sampling times are required to obtain desired MHb levels, it may be necessary to repeat the pilot study before dosing additional animals. If the pilot study is repeated, the same animals will be used after a sufficient wash-out period."

Reasons for Change: WRAIR has provided data and information for the compounds in question. Evaluation of the data may determine that a pilot experiment is not necessary, however, the option to conduct a pilot experiment should be maintained.

Impact on the Study: This change will not affect the integrity of the study. If the pilot study is not needed, this will minimize the number of animals used in the study and/or the number of exposures.

5. Change: Delete the first sentence after heading WR269410 on page 23 in the section Experimental Design - Phase VI: C. "Study Design", which reads:

"Compound WR269410 will be administered at 3 mg/kg by gavage every 8 hours for a 7-day period."

Substitute with the following:

"Compound WR269410 will be administered per os (dosage to be determined) every 8 hr for a 14-day period."

Reasons for Change: When the protocol was originally written, compound dosing was expected to be by oral gavage. A pilot study indicated that dosing of the compounds, particularly WR269410 in PEG 200, could be done by capsule. After discussions, WRAIR decided to change the number of dosing days to 14.

Impact on the Study: This change will not affect the integrity of the study. In previous in which this compound has been dosed, capsules have been found satisfactory. Dosing by capsule is believed to be the most favorable route of administration.

6. Change: Delete the second paragraph, on page 24, in the section Data Analysis: C. "Study Design", which reads:

"Analysis of the pharmacokinetic information for each drug is made after WRAIR has chemically analyzed the plasma and transferred data to Battelle. Drug data should be submitted to Battelle in a mutually acceptable format and time frame to

assist completion of the report. Analyses described for MHb and Hb data will be repeated for compound concentration data."

Substitute with the following:

"Analysis of the pharmacokinetic information for each drug will not be reported in the letter report for this phase of the study. The data may or may not be included as a letter report addendum for phase VI and consequently, the first draft. If the data are to be included in a report, then documentation of GLP compliance by the other facility performing the analysis will be included in the study file (by Quality Assurance Audit or other acceptable means). In addition, the drug data should be submitted to Battelle in a mutually acceptable format and time frame to assist completion of the report.

Reasons for Change: The Study Director, in discussion with the COR, has not determined whether the drug analyses data will be included in the letter report or final report for this study. If the plasma drug concentrations are reported during this study, then a Quality Assurance Audit of the facility analyzing the plasma samples is required for a Good Laboratory Practice study.

Impact on the Study: This change will not affect the integrity of the study.

Approval Signatures for Amendment No. 3

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Frances M. Reid — 6-1-94
Frances M. Reid, D.V.M. — Date
Study Director

David Stitcher — 6-2-94
David L. Stitcher, CIH — Date
MREF Environment, Safety and Health Officer

Don W. Korte Jr. LTC, MS — 2 Jun 94
LTC Don W. Korte, Jr., Ph.D. — Date
USAMRICD COR

10/16/94 KA

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Amendment No. 4 refers to Addendum 2:

Change: Delete the Phase VIII Experimental Design table in the section Experimental Design: C. "Study Design Phase VIII:", which reads:

PHASE VIII EXPERIMENTAL DESIGN

Animal	Period	
	1	2
1	Vehicle Control	PAHP
2	PAHP	Vehicle Control
3	Vehicle Control	PAHP
4	PAHP	Vehicle Control
5	Vehicle Control	PAHP
6	PAHP	Vehicle Control
7	Vehicle Control	PAHP
8	PAHP	Vehicle Control
9	Vehicle Control	PAHP

substitute with the following table:

PHASE VIII EXPERIMENTAL DESIGN

Animal	Period	
	1	2
1	PAHP	Vehicle Control
2	Vehicle Control	PAHP
3	PAHP	Vehicle Control
4	Vehicle Control	PAHP
5	PAHP	Vehicle Control
6	Vehicle Control	PAHP
7	PAHP	Vehicle Control
8	Vehicle Control	PAHP
9	PAHP	Vehicle Control

Reasons for Change: The dosing order was changed for the remainder of Phase VIII, based on the dosing order established by the Study Director on the dosing day.

Impact on the Study: This change will not affect the integrity of the study.

Approval Signatures for Amendment No. 4:

Frances M. Reid — 6-14-94
Frances M. Reid, D.V.M. — Date
Study Director

David Stitcher — 6-14-94
David L. Stitcher, CIH — Date
MREF Environment, Safety and Health Officer

Don W. Korte, Jr. LTC, MS — 14 Jun 94
LTC Don W. Korte, Jr., Ph.D. — Date
USAMRICD COR

6/16/94 KA

DEVIATION REPORT

SC930216

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Type of Deviation: (circle one): GLP / (PROTOCOL) / SOP

Date of Deviation: June 13, 1994

Nature of Deviation: The dosing order of PAHP and vehicle control was reversed from what was stated in the protocol. The animals received their correct dosage of compound, but the first animal should have received a vehicle control and the second animal PAHP.

Cause of Deviation: The first animal was dosed with the correct dosage of PAHP. However, the cross-over design required that the first dog be dosed with just vehicle control. The second dog was then dosed as the vehicle control for this dosing day. This resulted in a reversal of dosing order per the protocol.

Impact of Deviation on the Study:

There is no significant impact on the study. The cross-over design remains intact. The dosing order established by the first two animals will be maintained.

Corrective Action: An Ammendment to the protocol has been written to change the dosing order for Phase VIII to reflect the current dosing order.

Prepared By: Kara K. Audet Date: 6/14/94

Approved By: Frances M. Reed Date: 6-14-94
Study Director

Distribution: Study File (original),
LTC Korte,
Study Director (copy,
QAU Inspector (copy)

Initial Control Copy
6/16/94

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

MREF Protocol 98, Addendums 1 and 2, and Amendments 1-4.

Amendment No. 5 refers to Protocol 98, Phase 5 and Addendum 1.

Refers to Protocol 98

1. Change: Delete the second sentence of paragraph B. Test Material (2) Toxic-Agent on page 6, which reads:

"A saline dosing solution of 4 mg NaCN/mL is prepared daily prior to the initiation of that day's experiment(s)."

and substitute with the following:

"A saline dosing solution of approximately 4 mg NaCN/mL is prepared daily prior to the initiation of that day's experiment(s) for the applicable phases except Phase V. For Phase V, AN approximately 4 mg NaCN/mL stock solution may be prepared and analyzed for concentration during the week prior to dosing. Dosing aliquots of the stock solution may be prepared and a portion analyzed for CN concentration."

Reasons for Change:

Review of data from previous phases indicated variability in the concentration of CN in the dosing solutions. Consistency in the NaCN dosing solution is important in Phase V because of the fixed NaCN infusion time to deliver a fixed dose. Preparing a stock solution in advance and preparing dosing aliquots is expected to: 1) allow analysis of the dosing solution in advance, 2) provide greater accuracy in delivering a set dose as a result of knowing the concentration of the dosing solution, and 3) improve consistency in dosing between animals. In addition, data exist to support the stability of NaCN in a saline solution beyond the maximum 2 week requirement necessary to complete Phase V.

Impact on the Study:

This change will not affect the integrity of the study. Battelle chemists have data to support the stability of NaCN in a saline solution beyond the time of approximately 2 weeks required for Phase V.

2. Change: Delete the following sentence of paragraph C. (5) Phase V - EFFICACY OF MHb-INDUCING DRUGS IN COUNTERING THE EFFECTS OF NaCN ADMINISTERED AT TWICE THE DOSE CAUSING RESPIRATORY ARREST OF UNTREATED ANIMALS:

"Predicted therapeutic doses of the MHb formers are used to determine efficacy."

and substitute with the following sentence:

"A predicted methemoglobinemia level (within an acceptable range) or a compound dose may be used to determine efficacy."

Reasons for Change:

When the protocol was originally written, limited information was available for determining the best approach to assessing the efficacy of low methemoglobin levels in protecting against the lethal effects of cyanide at an estimated threat level. Data produced from this study have provided information on variability and prediction of MHb level from a given dose. Review of this information suggested that using a dose of MHb-former may or may not be the best method to achieve the purpose of this phase. The Study Director requires the flexibility to choose between compound dose or MHb level to determine efficacy.

Impact on the Study:

This change will not affect the integrity of the study. The Study Director gains flexibility in determining the best approach for methodology in determining efficacy . A memorandum to file signed by the Study Director and the COR will outline in greater detail the methodology of this Phase.

Refers to Addendum 1 Phase VI

3. Change: Delete the following paragraph of Addendum 1 on page 23 of Protocol 98:

"Should vomiting occur within 30 minutes of dosing, then the complete prescribed dose of the drug is re-administered. Vomiting after 30 minutes may preclude further blood sampling. This will be at the discretion of the Study Director ."

Substitute with the following:

"Should vomiting occur within 30 minutes of dosing, then the complete prescribed dose of the drug may, at the discretion of the Study Director, be readministered ."

Reasons for Change:

The original protocol was too specific on the issue of readministration of the MHb-former if vomiting occurred within 30 minutes after dosing. Situations have arisen during this experiment that indicate that readministration may not be advisable. Methemoglobinemia can be a life-threatening condition. The Study Director is concerned that clinical methemoglobinemia may be observed during the multiple-dosive experiment. In addition, the multiple dose experiment is attempting to target low levels of MHb. The change allows the Study Director to determine if readministration of the compound should occur. Decisions made by the Study Director in cooperation with the COR are included in the Study File.

Impact on the Study:

This change will not affect the integrity of the study. The change allows the Study Director to determine if readministration of the compound should occur.

4. Change: Delete the second sentence in Addendum 1 to Protocol 98 on page 24, first paragraph under Data Analysis.

Reasons for Change:

For pharmacodynamic/kinetic studies, the 0 minute blood sample can serve, at a minimum, as the baseline blood sample. An additional sample can be taken, but does not have to be at 1 minute prior to dosing. The frequency of sampling and amount of blood withdrawn is of concern to the Study Director. The -1 minute blood sample was not deemed necessary by the Study Director and COR.

Impact on the Study:

This change will not affect the integrity of the study. The 0 minute blood sample serves as a baseline blood.

Refers to Amendment No. 3

5. Change: Delete the substitute paragraph on page 35 of Amendment 3, number 6 change. The paragraph to be deleted is as follows:

"Analysis of the pharmacokinetic information for each drug will not be reported in the letter report for this phase of the study. The data may or may not be included as a letter report addendum for phase VI and consequently the first draft. If the data are to be included in a report, then documentation of GLP compliance by the other facility performing the analysis will be included in the study file (by Quality Assurance Audit or other acceptable means). In addition, the drug data should be submitted to Battelle in a mutually acceptable format and time frame to assist completion of the report."

Substitute with the following paragraph:

> "Battelle will collect plasma samples and send them to WRAIR or their designee for analysis. However, the data generated from the analysis of plasma samples are not to be a part of, or reported with this study, Task 9228, SC930216. In addition, the final report will only state that the samples w ere collected and sent to the study sponsor or his designee."

Reasons for Change:

The Study Director discussed issues regarding the analysis of plasma samples for each compound, issues of GLP compliance, and issues regarding reporting of results. After considerable discussion, the COR decided that the plasma samples for each compound would be provided to WRAIR or their designee to proceed with as they wish.

Impact on the Study:

The integrity of the study will not be affected. This change is expected to reduce the time necessary to complete the study.

Approval Signatures for Amendment No. 5

Frances M. Reid — 7-26-94
Frances M. Reid, D.V.M., M.S. — Date
Study Director

David L. Stitcher — 7/27/94
David L. Stitcher, CIH — Date
Safety and Surety Officer

Don W. Korte, LTC, MS — 29 July 94
LTC Don W. Korte, Jr., Ph.D. — Date
USAMRICD COR

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8/11/94 KA

MREF Protocol 98
SC930216
Medical Research and
Evaluation Facility
August 10, 1994

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Amendment No. 6 refers to Protocol 98.

1. Change: Delete the second sentence of the first paragraph under "11. C. Study Design (1) PHASE I - EFFECTS OF REPEATED INFUSION PLUS THERAPY IN ANESTHETIZED DOGS", stated as follows:

   "In Phase I, four dogs are anesthetized, catheterized, intubated and instrumented to record air flow, respiratory rate, and ECG."

   and replace with the following sentence:

   "In Phase I, four dogs are anesthetized, catheterized, intubated and instrumented to record physiological parameters ."

2. Change: Delete the last sentence of the first paragraph under "11. C. Study Design (1) PHASE I - EFFECTS OF REPEATED INFUSION PLUS THERAPY IN ANESTHETIZED DOGS", which reads:

   "Parameters to be measured and recorded include: blood CN (total and/or plasma) levels, Hb, percent MHb, rate of respiration, air flow, and heart rate."

   and replace with the following sentence:

   "Parameters that may be measured and recorded include: blood CN (total and/or plasma) levels, Hb, percent MHb, rate of respiration, air flow, and heart rate."

3. Change: Delete the following measurements under "11. E. Measurements Recorded":

   "Air Flow - tidal volume and/or minute volume Respiratory Rate"

   and substitute with the following:

   "Respiratory Parameters (in cluding respiratory rate)"

Reasons for Change:

The flexibility is needed to record the parameters that best describe the physiological events occurring during cyanide infusion and perform measurments within the capability of the PO-NE-MAH® Data Acquisition, Analysis, and Archiving System. During a December 29 1993 meeting with the client, the accuracy and sensitivity of the PO-NE-MAH® Data

Acquisition, Analysis, and Archiving System were discussed with the client. The extreme sensitivity associated with the respiratory parameters was explained to the client. The PO-NE-MAH® company suggested that alternate settings be tried or the software rewritten. Time constraints and the prolonged time necessary for additional validation did not make software rewriting a viable option. The client identified the primary parameters (time to respiratory arrest, amount of sodium cyanide administered, and percent methemoglobin) to be measured and parameters of secondary interest (heart rate, respiratory rate, and air flow).

Impact on the Study:

This change will not affect the integrity of the study. The primary parameters have been measured and reflect the needs of the client. The PO-NE-MAH® Data Acquisition, Analysis, and Archiving system has been validated within the limits specified in the validation package for specified heart rate and respiratory rate.

Approval Signatures for Amendment No. 6

Frances M. Reid — 8-10-94
Frances M. Reid, D.V.M., M.S. — Date
Study Director

David L. Stitcher — 8-11-94
David L. Stitcher, CIH — Date
Safety and Surety Officer

Richard R. Stotts — 17 Aug 94
LTC Richard R. Stotts, VC — Date
USAMRICD COR

MREF Protocol 98
SC930216
Medical Research and
Evaluation Facility
June 14, 1995

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Amendment No. 7

Change: Replace the incorrect Battelle SOP MREF VI-010 number at the end of the last sentence of paragraph two in section 11. A. Test System (12) Study Preparation on page 5 with the correct number Battelle SOP MREF VI-015.

Reasons for Changes:

The last digit of the SOP number was incorrect. The correct SOP number replaces the 0 with a 5, so that the correct number reads Battelle SOP MREF V I-015.

Impact on the Study:

There are no adverse affects on the study.

Approval Signatures for Amendment No. 7

Frances M. Reid — 6-14-95
Frances M. Reid, D.V.M., M.S. — Date
Study Director

David L. Stitcher — 6-14-95
David L. Stitcher, CIH — Date
Safety and Surety Officer

Richard R. Stotts — 14 JUN 95
LTC Richard R. Stotts, VC — Date
USAMRICD COR

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1/11/96 KA

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Protocol Amendment No. 8

Change 1: Page 1, Section 1.

Change to: "Co-Principal Investigator and Manager: John B. Johnson, D.V.M., Medical Research and Evaluation Facility (MREF)".

Reason for change:

The principal investigator and manager has changed.

Change 2: Page 1, Section 6. Study Veterinarian.

Change to: "Tracy A. Peace, D.V.M.".

Reason for change:

The study veterinarian has changed.

Change 3: Page 1, Section 7. Sponsor.

Change to: "U.S. Army Medical Research and Materiel Command (USAMRMC)".

Reason for change:

The name of the sponsoring organization has been changed.

Change 4: Page 1, Section 8. Sponsor Monitor.

Change to: "LTC Richard R. Stotts, D.V.M., Ph.D., U.S. Army Medical Research Institute of Chemical Defense (USAMRICD)".

Reason for change:

The sponsor monitor has changed.

Approved by:

Frances M. Reid
Frances M. Reid, D.V.M., M.S.
Study Director

11-9-95
Date

Richard R Stotts
LTC Richard R. Stotts, D.V.M., Ph.D.
USAMRICD COR

9 NOV 95
Date

DEVIATION REPORT

Study Number SC930216

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Type of Deviation: GLP Protocol

Dates of Deviation: Duration of Task 28

Nature of Deviation:

Animals were supplied water via automatic watering system instead of water bowls as stated in the protocol.

Nature of Deviation:

The wording in the protocol was intended to state that a water bowl or automatic watering system would be used.

Impact of Deviation on Study:

There is no significant impact on the study.

Prepared by:

Kandy K. Audet
Kandy K. Audet
Researcher

8/13/96
Date

Frances M. Reid
Frances M. Reid, D.V.M.
Study Director

8-13-96
Date

DEVIATION REPORT

SC930216

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

TYPE OF DEVIATION (circle one): **GLP / PROTOCOL / (SOP)**

DATE OF DEVIATION: September 19, 1996

NATURE OF DEVIATION: This study had changes made to the protocol by using ammendments. Additional phases were added to the protocol by using Addendums and detailed method of conduct for each phase was by study memorandums, which are not according to Battelle SOP No.: GEN.I-029-05; Battelle's Standard Operating Procedure for Preparation, Amendments, and Deviation Reports, effective date October 6, 1995.

CAUSE OF DEVIATION: When the study was initiated, the Quality Assurance (QA) representative and QA manager assisting the study director, approved the procedures described above. Since there has been a change in management prior to finalizing this study, the procedures have changed and this deviation is required by Battelle SOP No.: GEN.I-029-05; Battelle's Standard Operating Procedure for Preparation, Amendments, and Deviation Reports, effective date October 6, 1995.

IMPACT OF DEVIATION ON THE STUDY: There is no significant impact on the study.

CORRECTIVE ACTION: None necessary.

APPROVED BY: Frances McReid DATE: 9-19-96
STUDY DIRECTOR

Distribution: Study File (original), +copy
LTC Stotts (copy),
Study Director (copy),
Final Report (copy), and
QAU Inspector (copy)

APPENDIX B

STUDY FILE MEMORANDUMS

Project Number G1555-9001 (8846)
SC 93 0216

Battelle
. . . Putting Technology To Work

Internal Distribution

RG Menton
CT Olson
FM Reid
DW Hobson/Study File

Date September 27, 1993

To LTC Don W. Korte, Jr.

From Frances M. Reid

Subject Task 92-28, MREF Protocol 98

1. WR000302AG or PAPP

Data from pharmacodynamic studies for PAPP were provided by the U.S. Army Medical Research and Development Command (USAMRDC) and used to determine the recommended doses. The attached document provides the method for determining the recommended doses. These doses are:

Maximum % MHb	Recommended Doses mg/kg
2.5	0.10
5.0	0.13
10.0	0.22
15.0	0.37

PAPP is prepared in polyethylene glycol (PEG 200), according to the preparation document provided by the USAMRDC. The following are approximate target bleeding times recommended (within ± 1 minute):

In minutes - Baseline -5, 0, 5, 10, 20, 30, 60, 90, 120, 150, 180, 240, 300, 360, and 420.

If the methemoglobin level has not returned to within ± 2 percent MHb units of baseline, then additional blood withdrawals may be necessary. The hemoximeter requires a minimum of 0.3 mL for analysis.

2. WR242511AE

Data from pharmacokinetic/dynamic studies for WR242511AE were provided by USAMRDC and used to determine the recommended doses. The attached document provides the method for determining the recommended doses. These doses are:

Maximum % MHb	Recommended Doses mg/kg
2.5	1.3
5.0	2.5
10.0	5.0
15.0	7.4

The following are approximate target bleeding times recommended (within ± 1 minute):

In hours - Baseline -5, 0, 6, 9, 12, 15, 18, 24, 30, 48, 72, 96, 168, 216, 264, 336, 384, 432, 504, 552, and 600.

If the methemoglobin level has not returned to within ± 2 percent MHb units of baseline, then additional blood withdrawals may be necessary. The hemoximeter requires a minimum of 0.3 mL for analysis.

FMR/tsk

Attachments

Approved by: Frances M. Reid 9-27-93
Frances M. Reid, D.V.M., M.S.
Research Scientist

Don W. Korte LTC, MS
LTC Don W. Korte, D.V.M., MS, COR

Phase II of MREF Task 92-28 involves pharmacodynamic studies for two compounds: short- and long- acting methemoglobin formers. The U.S. Army conducted preliminary experiments (Protocol C001-92) to assess the pharmacodynamics of the short-acting drug (PAPP) for various routes of administration. A total of 22 dogs were administered PAPP IV without an anesthetic. Table 1 summarizes the data for these animals. Linear regression models were fitted to the maximum percent metHB (Cmax) for these animals using (1) concentration of PAPP and (2) log-concentration of PAPP as the independent variable. The regression model utilizing log-concentration of PAPP as the independent variable appeared to provide a better fit to the observed Cmax data. Therefore, the estimated parameters of the log-concentration regression model were employed to calculate the PAPP dosages predicted to yield 2.5, 5, 10, and 15 percent maximum metHB. Recommended doses of PAPP for Phase II experiments, displayed in Table 2, rounded off to two decimal places are 0.10, 0.13, 0.22, and 0.37 mg/kg.

Table 1. Mean and Standard Deviation of Maximum Percent MetHb for each PAPP dose group.

Papp Dose	Mean MetHb Level	Standard Dev.	n	Min	Max
0.1	3.300	0.566	2	2.900	3.700
0.2	6.700	0.141	2	6.600	6.800
0.3	13.813	2.627	8	11.000	19.400
	15.490	1.571	10	12.900	18.200

Table 2. Doses of PAPP Caculated to Yield Specific levels of Maximum Percent MetHb Based on Log-concentration Regression Model.

Maximum Percent MetHb	Predicted LN(PAPP)	Predicted PAPP	Geometric Standard Dev.	Lower 95% CI	Upper 95% CI
2.5	-2.323	0.098	1.304	0.056	0.171
5	-2.057	0.128	1.286	0.076	0.216
10	-1.526	0.217	1.263	0.134	0.354
15	-0.994	0.370	1.261	0.228	0.600

Phase II of MREF Task 92-28 involves pharmacodynamic studies for two compounds: short- and long- acting methemoglobin formers. The U.S. Army conducted preliminary experiments to assess the pharmacodynamics and pharmacokinetics of the long-acting drug (WR242511) for two routes of administration: oral and IV. Unfortunately only a single dose (7.0 mg/kg p.o.) was administered orally, and only two doses (3.5 mg/kg and 7.0 mg/kg IV) were administered via IV. Average maximum percent metHb observed at 7.0 mg/kg for the oral and IV doses were 13.9% and 15.6 %, respectively. Results at the two IV doses were utilized to estimate doses of WR242511 that produce 2.5, 5, 10 and 15 percent methemoglobinemia.

A total of 9 dogs were administered WR242511 IV. Table 1 summarizes the data for these animals. A linear regression model was fitted to the maximum percent metHB (Cmax) for these animals using concentration of WR242511 as the independent variable. Because only two doses of WR242511 were available, it was not possible to assess the appropriateness or adequacy of the fitted model; we should not place much confidence on doses estimated from this model. Estimated parameters of the regression model were employed to calculate the WR242511 dosages predicted to yield 2.5, 5, 10, and 15 percent maximum metHB. Recommended doses of WR242511 for Phase II experiments are displayed in Table 2.

Table 1. Maximum Observed Percent MetHb for each IV Test of WR242511.

Dose	Observed Max. % Methb	AVG	SD
3.5	2.6		
3.5	8.3		
3.5	10.1	7	3.92
7	18.8		
7	15.5		
7	14.4		
7	16.3		
7	13.2		
7	7.2	14.23	3.93

Table 2. Doses of WR242511 Caculated to Yield Specific Levels of Maximum Percent MetHb Based on Regression Model.

Maximum Percent MetHb	Predicted WR242511	Standard Dev.
2.5	1.32	2.58
5	2.53	2.33
10	4.95	2.03
15	7.37	2.08

Project Number G1555-9001 (8846)
SC930216

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Internal Distribution

T Hayes
LTC Korte
R Menton
C Olson
F Reid
QA
DW Hobson/File

Date October 4, 1993

To Task 92-28 Study File

From Frances M. Reid

Subject Task 92-28, MREF Protocol 98

1. Terminating Blood Collection.

   When the methemoglobin (MHb) level in whole blood has returned to within ± 1 percent MHb unit of baseline, then blood collection may be terminated. Collection of additional samples are not considered necessary for establishing the dose-response curve.

2. Solvent for PAPP

   WR000302AG (PAPP) solutions for intravenous administration will be formulated using a saline vehicle for the remainder of TASK 92-28, unless the Sponsor specifies otherwise. The assumption is that there is no difference in MHb effect due to the vehicle. PAPP administered in polyethylene glycol 200 (PEG 200) was observed to cause a local inflammatory response at the injection site, despite slow administration. PAPP is readily dissolved in saline and this solution should not cause a local inflammation.

Approved by:

Frances M. Reid — 10-4-93
Frances M. Reid, D.V.M., M.S. — Date
Research Scientist
Study Director

Don W. Korte, DVM, MS — 10/8/93
LTC Don W. Korte, M.S., COR — Date

Project Number G1555-9001 (8846)

Battelle
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Internal Distribution

FM Reid
LTC Korte
DW Hobson/File

Date October 13, 1993

To Study File

From Frances M. Reid

Subject Task 92-28, MREF Protocol 98 SC930216

1. Solvent for PAPP

WR000302AG (PAPP) solutions for intravenous administration will be in polyethylene glycol 200 (PEG 200) for the remainder of TASK 92-28. A 3.0 mg PAPP/mL solution could not be prepared in sterile saline. The Study Director decided to use the original solvent, PEG 200. A catheter for PAPP administration will be placed in the saphenous vein on the side opposite the jugular site used for blood withdrawal. PAPP administered in polyethylene glycol 200 (PEG 200) through a catheter and over a period of 1 minute did not appear to cause a local inflammatory response. It was decided, following a discussion with the contracting officer's representative, that PEG 200 will be the vehicle to use for PAPP. An approximately 1 mL volume of saline will be used to flush the PAPP from the catheter.

Approved by:

Frances M. Reid — 10-13-93
Frances M. Reid, D.V.M., M.S. — Date
Research Scientist
Study Director

Don W. Korte, LTC, MS — 14 OCT 93
LTC Don W. Korte, M.S., COR — Date
USAMRICD

Project Number G1555-9001 (8846)

Battelle
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Internal Distribution

LTC Korte
CT Olson
FM Reid
RMO
QA
DW Hobson/File

Date October 28, 1993

To Study File SC930216

From Frances M. Reid

Subject Bleeding Times for WR242511AE and Replacement for Animal HFXBDB

1. Bleeding Times for WR242511AE

   In reviewing the results of the methemoglobin levels obtained to date, a change in the bleeding schedule is proposed to the U.S. Army Contracting Officer's Representative (COR). The results indicated induction of methemoglobinemia by WR242511AE continues to rise on day two. The Study Director proposes taking daily blood samples for as long as the level of methemoglobin continues to rise. Once a plateau has been reached, a Monday, Wednesday, Friday blood collection schedule would be accomplished for at least one week. When the methemoglobinemia is observed to be in the elimination phase (downward slope of the curve), a weekly blood sample for methemoglobin level is obtained. The amount of blood necessary for analysis for methemoglobin is less than 0.4 mL. The remaining experimental periods for this phase will follow a blood collection schedule established for this period (Period 1 of Phase II for WR242511AE).

2. Replacement of Animal HFXBDB With Animal HFXBGH In Phase II For WR000302AG

   Prior to administering the second dose on October 6, 1993, animal HFXBDB died from an adverse reaction to sodium pentobarbital. Discussion with LTC Korte resulted in a decision to replace this animal since only one day's dosing results had been obtained. Animal HFXBER was randomly chosen from the list of remaining unassigned dogs. After receipt at the MREF, the Study Director determined that HFXBER could not be placed on study at that time. The animal was approximately 10% dehydrated and required nutritional supplementation to improve physical appearance (possibly related to stress). An alternate animal was randomly selected for the replacement. HFXBGH was used in Period 1 of Phase II for PAPP on October 13, 1993 and will continue on the dosing regimen originally established for HFXBDB.

Approved by:

Frances M. Reid — 10-28-93
Frances M. Reid, D.V.M., M.S. — Date
Research Scientist
Study Director

Don W. Korte Jr, MS — 28 OCT 93
LTC Don W. Korte, M.S., COR — Date

Date: November 8, 1993

To: Study File, Task 28, SC930216

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Subject: Memorandum for the Record

Events that occur on study that are not planned, and discussion of their possible impact on the results of the study, are recorded periodically.

Phase II - Limited Methemoglobin Kinetic Studies with Two Compounds.

1. WR242511AE

a. 11-2-93 An additional blood sample was collected from each animal assigned this compound at their designated bleeding time on this date.

Impact: Since methemoglobin levels continued to increase through October 29, 1993, the study statistician and the study director decided that an additional blood sample should be drawn on Tuesday, Nov. 2, 1993 (192-hr sample). The Army's Contracting Officer's Representative agreed. There are no adverse effects on the results of this phase of the study.

b. The 336 hour blood sample collection was not obtained within the approximate 1 minute window usually sufficient for blood collection due to difficulty with calibrating the OSM™3 Hemoximeter. This proved to be technician error rather than failure of the instrument. The following list contains the differences in time in actual blood collection for each animal.

11-8-93	HFYBEI	336 hour blood sample was actually obtained 33 min later than scheduled.
	HFXBGI	336 hour blood sample was actually obtained 25 min later than scheduled.
	HFZBFI	336 hour blood sample was actually obtained 17 min later than scheduled.

Impact: Pharmacodynamic analyses are based on the actual time the blood was collected, and therefore there are no adverse effects on the study results.

Approved By:

Frances M. Reid
Frances M. Reid, D.V.M., M.S.
Study Director

11-8-93
Date

Don W. Korte, Jr. LTC, MS
LTC Don W. Korte, Jr., Ph.D.
USAMRICD COR

8NOV93
Date

Date: November 29, 1993

To: Study File

Copy: LTC Don Korte
Frances Reid
Carl T. Olson
Ron Menton
David W. Hobson

Subject: Dosing of WR242511AE and Bleeding Times for Phase II: A Limited Pharmacokinetic/dynamic Study and Phase I Recommended Bleeding Times: Repeated Cyanide Dosing To Effect.

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Task 28, Protocol 98, SC930216

1. WR242511AE

Following the completion of the first period of dosing WR242511, I recommend the following target bleeding times (within ±1 minute):

In hours - Baseline (-5 min, 0 hr) 6, 9, 24, 48, 72, 96, 120, 144, 168, 192, 216, 264, 336, 432, 504, and 600.

If the methemoglobin level has not returned to 2 % or less, then additional blood withdrawals may be necessary. If the methemoglobin level is 2 % or less, then the sample collection may be stopped by the study director.

2. First Period Dosing of WR242511AE

During preparation of animals for dosing (body weights and preparing samples), Animals 3 and 4 were switched. Animal 3, HFZBFI, was administered 5.0 mg/kg of WR242511AE during the first period of dosing. Animal 4, HFYBCP, was administered 7.4 mg/kg of WR242511AE during the first period of dosing. According to the randomization sheet, HFZBFI should have received the 7.4 mg/kg dose and HFYBCP should have received the 5.0 mg/kg dose. Since each dog's dose was calculated for his weight, the two dogs will be switched in position for the remainder of Phase II dosing of WR242511AE. Animal HFZBFI is now animal 4 and HFYBCP is now animal 3.

Impact on Study - This will not have any impact on the study. The animals received the correct amount of compound for their body weight. By switching position numbers for these animals, the protocol can continue as stated. Since the animals were selected by a computer randomization program initially, randomization has not been compromised.

3. Recommended blood sampling for Phase I

The following is recommended for sampling blood during Phase I:

> In minutes - -5, -1, CN Infusion (no blood draws), 1 min post-CN infusion and every 1 minute thereafter until CN infusion stops, 1 minute after CN infusion stops and then every 2 minutes until 9 minute following cessation of CN infusion. Samples will be then taken at 15 min, 20 min and 30 minute after cessation of CN infusion, then every 30 minutes for up to 2 hours following cessation of CN infusion.

Note: The above blood sampling schedule may be modified depending on observations during dosing days. A minimum of two samples during CN infusion will be obtained and a minimum of 10 samples will be obtained after CN infusion has stopped. A 3-mL blood sample is needed to analyze for both whole and plasma CN through the third sample after stopping CN infusion (5 min following cessation of infusion). The remaining blood samples will only require 1 mL of blood for whole blood CN analysis.

Approved By:

Frances M. Reid — 11-29-93
Frances M. Reid, D.V.M., M.S. — Date
Study Director

Don W. Korte — 29 Nov 93
LTC Don W. Korte, Jr., Ph.D. — Date
USAMRICD COR

Date: December 2, 1993

MEMO TO STUDY FILE: SC930216 Task 9228

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Subject: Measurement of strip chart recordings.

DATA EVALUATION AND PRESENTATION

I. ANALYSIS AND EVALUATION OF ASTRO-MED STRIP CHART RECORDER

The Astro-Med strip chart record of respirations and heart beats is evaluated at blood collection time points, major events and other time points deemed relevent by the Study Director. The time interval, over which the data are evaluated, may vary depending on the parameter (heart rate or respiratory rate) being measured and what effect is observed. A calibrated ruler is used to measure the time intervals, distance between time intervals (where necessary), distance between beginning cyanide (CN) infusion and ending CN infusion. The breaths or beats are then counted within that time interval and multiplied by the appropriate factor (for that time interval) to determine the number of breaths or beats per minute. The data from the recorder and the data averaged over the same time frame from the PO-NE-MAH™ printout are recorded on the attached form.

Phase I

1. Strip Chart Evaluation of Heart Rate

The time interval over which heart rate was determined in Phase I was a 5 second interval. The periods of analysis included at a maximum the following approximate times:

Baseline	-	-5 minute, -4 minute, and -1 minute prior to CN infusion.
Events	-	Begin CN Infusion, End CN Infusion, every blood collection event through and one blood collection past Hydroxylamine therapy,.
Other	-	Every twenty seconds between blood samples or nearest blood sample, or as determined by the study director.

2. Strip Chart Evaluation of Respiratory Rate

The time interval over which respiratory rate was determined in Phase I was a variable time interval. Generally, a 5 second time interval was used for baseline analyses periods and variable time intervals for intervals around the time of respiratory arrest. The periods of analysis included at a maximum the following:

Baseline	-	-5 minute, -4 minute, and -1 minute prior to CN infusion

Events	-	Begin CN Infusion, End CN Infusion, every blood collection event through and one blood collection past Hydroxylamine therapy
Other	-	Every twenty seconds between blood samples or nearest blood sample, or as determined by the study director.

3. Strip Chart Evaluation of Time to Respiratory Arrest

Measurement began at the beginning of the "Begin Cyanide Infusion" event marker and was measured, using a calibrated ruler, to the event marker labeled "End Cyanide Infusion".

Frances M. Reid 12-2-93
Frances M. Reid,
Study Director

Date: January 12, 1994

MEMO TO STUDY FILE: SC930216 Task 9228

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Subject: Measurement of strip chart recordings.

DATA EVALUATION AND PRESENTATION

I. ANALYSIS AND EVALUATION OF ASTRO-MED STRIP CHART RECORDER

The Astro-Med strip chart record of respirations and heart beats is evaluated at blood collection time points, major events and other time points deemed relevent by the Study Director. The time interval, over which the data are evaluated, may vary depending on the parameter (heart rate or respiratory rate) being measured and what effect is observed. A calibrated ruler is used to measure the time intervals, distance between time intervals (where necessary), distance between beginning cyanide (CN) infusion and ending CN infusion. The breaths or beats are then counted within that time interval and multiplied by the appropriate factor (for that time interval) to determine the number of breaths or beats per minute. The data from the recorder and the data averaged over the same time frame from the PO-NE-MAH™ printout are recorded on the attached form.

Phase III.

1. Strip Chart Evaluation of Heart Rate

The time interval over which heart rate was determined in Phase III was a 5 second interval. The periods of analysis included at a maximum the following approximate times:

Baseline	-	-1 minute prior to PAPP administration, PAPP administration, and -1 minute prior to CN infusion,
Events	-	Begin CN Infusion, End CN Infusion, every blood collection event through and one blood collection past Hydroxylamine therapy,
Other	-	Or as determined by the study director.

2. Strip Chart Evaluation of Respiratory Rate

The time interval over which respiratory rate was determined in Phase III was a variable time interval. Generally, a 5 second time interval was used for baseline and early CN infusion time periods and variable time intervals for intervals around the time of respiratory arrest. The periods of analysis included at a maximum the following:

Baseline	-	-1 minute prior to PAPP administration, PAPP administration, and -1 minute prior to CN infusion,
Events	-	Begin CN Infusion, End CN Infusion, every blood collection event through and one blood collection past Hydroxylamine therapy
Other	-	Or as determined by the study director.

3. Strip Chart Evaluation of Time to Respiratory Arrest

Measurement began at the beginning of the "Begin Cyanide Infusion" event marker and was measured, using a calibrated ruler, to the event marker labeled "End Cyanide Infusion".

Frances M. Reid 1-2-94
Frances M. Reid,
Study Director

Date: January 14, 1994

To: Study File, Task 28, SC930216

Copy: LTC Korte
FM Reid
CT Olson
DW Hobson

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Subject: Memorandum for the Record

Events that occur on study that are not planned, and discussion of their possible impact on the results of the study, are recorded periodically.

Phase III Recommendations:

One animal per day will be challenged and we will run the first 4 days of the week.

1. DOSES of PAPP

Dose ID	Methemoglobin Level %	Dose mg/kg
A	0.0	0.0
B	2.5	0.12
C	5.0	0.17
D	10.0	0.37

PAPP will be administered through a catheter placed in a vein (saphenous vein through which the cyanide will be administered). Every attempt will be made to catheterize the saphenous vein opposite the jugular catheter inserted for blood collection.

2. DOSING TIME of PAPP and SODIUM CYANIDE (NaCN) ADMINISTRATION

a. PAPP will be administered after the animal has been anesthetized and at a minimum catheterized. Immediately following PAPP dosing, approximately 3 mL of a saline flush will be used to clear the catheter. Instrumentation of the animal may or may not be complete, since data from Phase II and Phase I indicate that the estimated pretreatment time may vary depending on level of methemoglobin desired. The following is the approximate time of CN infusion after the administration of PAPP:

Dose ID	Dose of PAPP mg/kg	PreCN Infusion Time min
A	0.0	28
B	0.12	28
C	0.17	37
D	0.37	56

b. NaCN will be infused at the time the predicted MHb level is estimated to be reached based on results obtained in Phase II (see 2.a. above for times). A blood sample will be collected at this time to determine the methemoglobin level and cyanide (CN, free and total) values.

3. BLOOD SAMPLING TIMES

The following are the recommended blood sampling times:

EVENT	BLOOD DRAW TIME (min)	AMOUNT (mL)	CHEM NOS
Baseline: One minute prior to administering PAPP a blood sample will be taken for CN analysis, tHb and MHb.			
Administer PAPP or Vehicle			
PreCN Infusion	- 1 prior to CN	3	
Pre CN Infusion	0	3	
Begin CN Infusion			
CN Infusion	1	3	
CN Infusion	2	3	
CN Infusion	3	3	
etc			
Continue 1 min, 3 mL blood drawls if CN Infusion continues			
Stop CN Infusion	Begin blood w/drawl	3	
Administer Hydroxylamine 30 sec after stopping CN infusion			
Post CN Infusion	1	3	
Post CN Infusion	5	3	
Post CN Infusion	10	1	
Post CN Infusion	15	1	
Post CN Infusion	30	1	
Post CN Infusion	60	1	

4. DATA ANALYSES

Data parameters to be collected for Phase III - Time to Respiratory Arrest, Dosage of CN, Manually calculated heart rate and respiratory rate, minute volume and cyanide analyses (free and total). The heart rate and respiratory rate parameters will be manually calculated at each blood draw time point during animals instrumentation and major event until hydroxylamine is administered.

5. DOSING OF HYDROXYLAMINE

The hydroxylamine therapy will be given through the cephalic catheter placed for administration of anesthesia. I believe this may help expedite the animals recovery, since administration site is closer to vital centers.

Frances M. Reid — 1-14-94

Frances M. Reid, M.S., D.V.M.
Study Director

Date

Don W. Korte, Jr. LTC, MS — 14 JAN 94

LTC Don W. Korte, Jr., PhD.
USAMRICD, COR

Date

SC930216 Task 9228 Protocol 98

Date: April 1, 1994

To: Study File, Task 28, SC930216

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Subject: Memorandum for the Record - Phase IV Methodology

Phase IV: Comparison of Two Methemoglobin-Forming Compounds at a Similar Methemoglobin Level (5 Percent).

1. Cyanide Analyses - Blood samples are analyzed only for total cyanide levels. Measurement of free cyanide is discontinued for the remainder of this study.

2. MHb Target Level - The level of methemoglobin (MHb) targeted in Phase IV for both MHb-forming compounds is 5%. For Period 3 experiments with WR242511AE, a range is established around 5% MHbemia, based on the Phase IV-Period 2 and Phase III data. Animals may be infused with CN once their measured MHb values fall within this range. Blood samples are collected -1 min and at the time of CN infusion for measurement of MHb, hemoglobin (Hb) values and total CN analyses.

3. Washout Time Periods - A minimum of one work-week washout between exposures is required for each animal.

4. Blood Sampling - Approximately 1.5 mL of whole blood is collected for samples analyzed for Hb, MHb and total CN levels. Baseline blood samples prior to pretreatment are not be taken during this phase. Blood sampling timepoints used in Periods 1 and 2 are based on those employed in Phase III for the A (0.0 mg/kg) and C (0.17 mg/kg) doses, respectively. For Period 3, baseline blood samples are taken within 24 hours of expected CN infusion for analysis of Hb and MHb only. The latter blood sample may assist in determining when the animal should be considered for CN infusion. Additional blood samples may be collected at sampling times prior to CN infusion based on Phase II results for WR242511AE and the inital blood sample taken at 3 days post WR242511AE administration. Baseline blood samples for analysis of Hb, MHb and Total CN are collected at same timepoints used in Periods 1 and 2. Blood sampling timepoints post CN infusion are the same as those utilized in Period 2 of Phase IV. A schedule for blood sampling timepoints for each period is attached .

5. Method for Performing Period 1 of Phase IV - Methods utilized in Period 1 are similar to those employed for the A dose experiments in Phase III. Length of time between vehicle dosing and CN infusion is identical to that established in Period 2 for PAPP experiments.

6. Method for Performing Period 2 of Phase IV - A constant time of 37 min is maintained between administration of PAPP and start of CN infusion. PAPP is administered I.V. at a dose of 0.20 mg/kg.

7. Method for Performing Period 3 of Phase IV, WR242511AE - Animal dosing for WR242511AE pretreatment may be staggered. As discussed above under Item 2, animals are infused with CN once their measured MHb values fall within the specified range of MHb levels. The order of animal dosing for CN infusion may be different from that used for WR242511AE pretreatment depending on variability in uptake of MHb from animal-to-animal. Phase II data for WR242511AE indicated that the range of peak levels of percent MHb (6.10 - 11.4 % MHb) were above 5 % MHb for each experiment at the 2.5 mg/kg dose and that MHb levels decreased to approximately 5 % within 4 to 8 days after pretreatment. Therefore, dose of WR242511AE employed in Period 3 experiments is 2.5 mg/kg.

Approved By:

Frances M. Reid — 4-1-94

Frances M. Reid, D.V.M., M.S.
Study Director — Date

Don W. Korte Jr MS — 14 APR 94

LTC Don W. Korte, Jr., Ph.D.
USAMRICD COR — Date

BLOOD SAMPLING TIMES PHASE IV

PERIODS 1 and 2

EVENT	BLOOD DRAW TIME (min)	AMOUNT (mL) Approximately	CHEM NOS
Administer PAPP or Vehicle			
PreCN Infusion	- 1 prior to CN	1.5	
Pre CN Infusion	0	1.5	
Begin CN Infusion			
CN Infusion	1	1.5	
CN Infusion	2	1.5	
CN Infusion	3	1.5	
etc			
Continue 1 min, approximately 1.5 mL blood drawls if CN Infusion continues			
Stop CN Infusion	Begin blood w/drawl	1.5	
Administer Hydroxylamine	**30 sec after stopping CN infusion**		
Post CN Infusion	1	1.5	
Post CN Infusion	5	1.5	
Post CN Infusion	10	1.5	
Post CN Infusion	15	1.5	
Post CN Infusion	30	1.5	
Post CN Infusion	60	1.5	

BLOOD SAMPLING TIMES PHASE IV

PERIOD 3

EVENT	BLOOD DRAW TIME (min)	AMOUNT (mL) Approximately	CHEM NOS
Administer WR242511AE			
3 days after WR242511AE administration take blood sample (0.5) mL for Hb and MHb analyses.			
PreCN Infusion	- 1 prior to CN	1.5	
Pre CN Infusion	0	1.5	
Begin CN Infusion			
CN Infusion	1	1.5	
CN Infusion	2	1.5	
CN Infusion	3	1.5	
etc			
Continue 1 min, approximately 1.5 mL blood drawls if CN Infusion continues			
Stop CN Infusion	Begin blood w/drawl	1.5	
Administer Hydroxylamine 30 sec after stopping CN infusion			
Post CN Infusion	1	1.5	
Post CN Infusion	5	1.5	
Post CN Infusion	10	1.5	
Post CN Infusion	15	1.5	
Post CN Infusion	30	1.5	
Post CN Infusion	60	1.5	

Date: May 6, 1994

To: Study File, Task 28, SC930216 Protocol 98

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Subject: Memorandum for the Record - A Pilot Study to Determine the Bioavailability (for methemoglobin (MHb) concentration in whole blood) of WR269410 (PAHP) administered per os.

Events that occur on study that are not planned, and discussion of their possible impact on the results of the study, are recorded periodically. Information provided by the U.S. Army indicated that capsule administration of PAHP in dogs did not result in MHb formation. Discussion among the U.S. Army's Contracting Officer Representative (LTC Korte); MREF's Chemist (Dr. Tim Hayes); and the Study Director (Dr. Frances Reid) lead to the decision to dose two dogs orally by capsule and monitor the hemoglobin and MHb level in whole blood over a specified time period. The pilot design is as follows:

Animals: Randomly chose 2 dogs from Phase III animals. Obtain a hematocrit and body weight within 24 hours of dosing. Remove feed no less than 12 hours before dosing.

Drug: PAHP - Bottle number BM08586 1 gm bottle. Chemistry will perform solubility studies in PEG200 and determine if PEG200 may be administered by capsule. Each animal's body weight will be used to determine the dose. The dose to be administered is 6 mg PAHP/kg body weight. One animal may receive a capsule of the crystalline/powder form and the other animal may receive a capsule of PAHP dissolved in PEG200.

Blood Collection Times: In minutes - 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 360, 420, 480, and 24 hours later. These times may be adjusted as needed. Approximately 0.5 mL of heparinized blood will be the most needed for MHb and Hb analysis.

Blood Analysis: Hb and MHb only.

Frances M. Reid 5-6-94

Date: May 18, 1994

To: LTC Don Korte

Copy: Study File, Task 28, SC930216
Dr. Frances Reid
Mr. Tim Hayes
Dr. Carl Olson
Ms. Jane Voller

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Subject: Request for additional test compound, WR269410.

1. The dosing information we currently have, indicates additional test compound, WR269410 (PAHP), will be required to complete Phases VI, VII, and VIII. This information is based upon data provided by Major Marino, and data we generated while performing the pilot study. We have estimated the amount of compound we would need based upon a dose of 6 mg PAHP/kg with an animal weight of 16 kg. The amount estimated to perform the dosing and the chemistry necessary to support this effort is 7 grams. Since this is a GLP study, it would be best to have 7 grams from the same lot to complete the remaining phases involving PAHP. We currently have 2 grams of material and will need at a minimum an additional 5 grams of PAHP preferably from the same lot as the PAHP we already have.

Requested By:

/Frances M. Reid

Frances M. Reid, D.V.M., M.S.
Study Director

5-18-94
Date

VERIFIED EXACT COPY
Frances M. Reid 5-18-94

Project Number G1555-9001 (8846)

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DW Korte
FM Reid
MREF File

Date June 1, 1994

To Task 92-28 Study File, SC930216

From Frances Reid

Subject Memorandum for the Record - Phase VI
Methodology for WR242511AE

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Phase VI: A ~~Limited~~ Multiple Dose and Pharmacodynamic and Pharmacokinetic Experiment with Multiple Doses of Compound WR242511AE.

WR242511AE

1. Test system - Animals used on Phase VI are those from Phases I and II. Six of these animals are randomly assigned to each treatment compound. As with previous phases, a hematocrit and body weight are obtained within 48 hr of dosing. Feed is removed no less than 12 hr before dosing.

2. Test compound - WR242511AE is administered orally in capsules similar to the method of dosing in previous Phases of Task 92-28 (Phases II and IV). A single dose level has been recommended.

   WR242511AE dosing regimen: A loading dose of 2 mg/kg on Day 0, followed by a 1 mg/kg maintenance dose every 48 hr for a minimum of two weeks.

   References to readministration of capsules, adjustments to schedules, dosing, dosing intervals, and samples collected are found in Protocol 98, Addendum 1 and Amendment 3.

3. Blood Collection - Approximately 2 mL of heparinized blood is collected for hemoglobin (Hb), methemoglobin (MHb), and drug analyses. Additional blood samples may be taken based on results for MHb and Hb analyses. Blood samples are collected at the following times (plus or minus several minutes); **A** refers to sampling after the initial dose, **B** refers to every other day blood sampling times after dosing, **C** refers to the blood sampling times following the last dose, and **0** represents the blood sample just prior to dosing.

   Drug and Hb/MHb blood samples, unless specified otherwise:

   A: In minutes - 0, 30, 60, 90, 120, 180, 240, 360, and 480.

   B: In hours - 0, 6, 12, and 24 (Hb and MHb only).

   C: In minutes - 0, 30, 60, 90, 120, 180, 240, 360, and 480.

Blood samples for Hb and MHb measurement are analyzed on the Radiometer Hemoximeter as soon as possible after sample collection. The remainder of the sample is centrifuged and plasma collected. For blood samples analyzed for Hb and MHb only, approximately 0.5 mL is collected. Dosing of WR242511AE is every 48 hr after the initial dose for two weeks; however, blood sampling for MHb and Hb values may be continued at various times for up to four weeks if the Study Director and Contracting Officer's Representative (COR) agree to the necessity.

4. WR242511AE Analyses - The plasma samples collected are stored at approximately -70 degrees C until the end of this study (after PAHP dosing and sample collecting are completed). The plasma samples are then shipped to Walter Reed Army Institute of Research or their designee for compound analyses.

5. Washout Time Periods - A minimum of one four-week washout following previous study exposure is required.

6. Other Issues - The COR and Study Director will review the data (MHb only) on a timely basis and make any necessary adjustments to doses or times. Depending on results or evaluation by the Study Director of animals, the experiment may be terminated by the Study Director following consultation with the COR.

Approved By:

Frances M. Reid — 6-1-94

Frances M. Reid, D.V.M., M.S.
Study Director

Date

Don W. Korte LTC, MS — 2 Jun 94

LTC Don W. Korte, Jr., Ph.D.
USAMRICD COR

Date

Project Number G1555-9001(8846)

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Date June 8, 1994

To Study File, Task 28, SC930216

From Frances M. Reid

Subject Memorandum for the Record - Phase VIII Methodology

Phase VIII: A Comparative Efficacy Experiment of a Methemoglobin-Former

1. Test Compound - Vehicle Control (PEG200 in a gelatin capsule) and WR2694210.

2. Animals Used - The animals assigned to Phase IV are to be used for Phase VIII.

3. Cyanide (CN) Analyses - Blood samples are analyzed for total cyanide levels only.

4. MHb Target Level - The level of methemoglobin (MHb) targeted in Phase IV for both MHb-forming compounds was 5%. This same MHb level will be targeted in Phase VIII. For WR269410, a range is established around 5% methemoglobinemia, based on the Phase IV data and Phase VII data. Animals may be infused with CN once their measured MHb values fall within this range. Baseline blood samples are collected -1 min and at the time of CN infusion for measurement of MHb, hemoglobin (Hb) values, and total CN analyses. Additional blood samples may be taken to assist in determining the targeted MHb range. Data from Phase VII will assist in determining the PAHP estimated time delay until CN challenge.

5. Washout Time Periods - A minimum of one work-week washout between exposures is required for each animal.

6. Blood Sampling - Approximately 1.5 mL of whole blood is collected for samples analyzed for Hb, MHb, and total CN levels. Baseline blood samples prior to pretreatment are not be taken during this phase. Blood sampling time points for the Vehicle Control are based on those employed in Phase IV for the A (0.0 mg/kg) dose. For WR269410, blood samples are based on Phase VII PAHP data. Additional blood samples may be collected for MHb and Hb only or may include total CN if data indicate the necessity. Baseline sampling points are those mentioned in Number 4 above. A schedule for blood sampling time points for each period is attached.

7. Method for Performing Vehicle Control Dosing in Phase VIII - Methods utilized for Vehicle Control (PEG200 in capsules) in Phase VIII are similar to those employed for the A dose experiments in Phase IV. Length of time between vehicle dosing and CN infusion is identical to that established for PAHP experiments, based on Phase VII data.

8. Method for Performing PAHP Dosing of Phase VIII - Based on Phase VII data, there will be approximately a 4-hr interval between administration of PAHP and the start of the CN infusion, and the dose of PAHP will be 5 mg/kg dissolved in PEG200 and administered by capsule. The interval between PAHP administration and CN challenge and the dose of PAHP will be finalized after review of the Phase VII data by the statistician. Blood samples for MHb and Hb only may be taken beginning at 1 hour prior to CN infusion to determine if the MHb blood level of the animal is within the 3% to 7% MHb range.

Approved By:

Frances M. Reid — 6-8-94
Frances M. Reid, D.V.M., M.S. — Date
Study Director

Don W. Korte, Jr. LTC, MS — 8 Jun 94
LTC Don W. Korte, Jr., Ph.D. — Date
USAMRICD COR

SC930216 Task 92-28 Phase VIII

BLOOD SAMPLING TIMES PHASE VIII

Vehicle Control

EVENT	BLOOD DRAW TIME (min)	AMOUNT (mL) Approximately	CHEM NOS
Administer Vehicle			
PreCN Infusion	- 1 prior to CN	1.5	
Pre CN Infusion	0	1.5	
Begin CN Infusion			
CN Infusion	1	1.5	
CN Infusion	2	1.5	
CN Infusion	3	1.5	
etc			
Continue 1 min, approximately 1.5 mL blood drawls if CN Infusion continues			
Stop CN Infusion	Begin blood w/drawl	1.5	
Administer Hydroxylamine 30 sec after stopping CN infusion			
Post CN Infusion	1	1.5	
Post CN Infusion	5	1.5	
Post CN Infusion	10	1.5	
Post CN Infusion	15	1.5	
Post CN Infusion	30	1.5	
Post CN Infusion	60	1.5	

BLOOD SAMPLING TIMES PHASE VIII

WR269410

EVENT	BLOOD DRAW TIME (min)	AMOUNT (mL) Approximately	CHEM NOS
Administer WR269410			
Approximately 3 hours after WR269410 administration take blood sample (0.5) mL for Hb and MHb analyses.			
PreCN Infusion	- 1 prior to CN	1.5	
Pre CN Infusion	0	1.5	
Begin CN Infusion			
CN Infusion	1	1.5	
CN Infusion	2	1.5	
CN Infusion	3	1.5	
etc			
Continue 1 min, approximately 1.5 mL blood drawls if CN Infusion continues			
Stop CN Infusion	Begin blood w/drawl	1.5	
Administer Hydroxylamine 30 sec after stopping CN infusion			
Post CN Infusion	1	1.5	
Post CN Infusion	5	1.5	
Post CN Infusion	10	1.5	
Post CN Infusion	15	1.5	
Post CN Infusion	30	1.5	
Post CN Infusion	60	1.5	

Project Number G1555-9001 (8846)

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FM Reid
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Date June 14, 1994

To Task 92-28 Study File

From Frances M. Reid

Subject Phase VIII Dosing and Modifications

1. Progress To Date: On June 13, 1994, animal HFYATS was dosed with WR269410 (PAHP) at 5.0 mg/kg and animal HFZANH was dosed with the vehicle control. Blood samples were taken for hemoglobin and methemoglobin (MHb) analysis, beginning approximately 1.5 hours after dosing. The MHb level indicated the PAHP-dosed animal was within the CN challenge range of 3.0 - 7.0 percent MHb (3.7 percent). Additional blood samples (approximately 30 minutes apart) indicated that HFYATS remained above the minimum 3.0 percent MHB level. HFYATS was prepared for CN challenge and the -1 min blood sample taken. The MHb level had dropped to 2.6 percent. The Study Director stopped the experiment for this dog. The second dog was infused with CN approximately 3 hours after dosing with the vehicle control. On June 14, 1994, animal HFYAJM was dosed with WR269410 (PAHP) at 5.0 mg/kg and animal HFZAGX was dosed with the vehicle control. The capsule for HFYAJM appeared to have leaked around the seam. This was noted on the dosing sheet. Blood samples were taken for hemoglobin and MHb analysis, beginning approximately 1.0 hour after dosing for the PAHP-dosed animal. The MHb level was at 2.0 percent MHb. An additional blood sample (approximately 30 minutes apart) indicated that HFYAJM was at 2.2 percent. A two-hour blood sample indicated that the MHb level had dropped to 1.1 percent. The experiment was stopped. The Study Director consulted with LTC Korte concerning the two initial days of dosing.

   The stock dosing solution for PAHP was prepared at an expected concentration of 120 mg/mL in polyethylene glycol. Dosing concentration confirmation of the stock solution indicated that the solution was 105 percent of expected concentration.

2. Consultation with LTC Korte: The two days of dosing Phase VIII were discussed. The following actions were decided:

   a. On Tuesday, June 14, 1994, choose two animals from Phase VII and dose with 5.0 mg/kg PAHP. Blood samples for MHb levels will be taken at half hour intervals, beginning at one hour post-dosing through approximately 4.5 hours post-dosing. These values will be compared to the animals' previous MHb levels. Note: Food from these animals will not have been removed the previous day for dosing as was done in Phase VII.

   b. On Wednesday, June 15, 1994, dose the next two animals according to protocol and memo to file dated June 8, 1994. If the PAHP-dosed animal responds as expected to the PAHP dose (a MHb level of approximately 5 percent), continue the study as designed. If the PAHP-dosed animal does not respond as expected (MHb values of at least 4 percent), stop the experiment and reevaluate with LTC Korte and Battelle staff.

Approved By:

Frances M. Reid 6-14-94
Frances M. Reid, D.V.M., M.S. Date
Study Director

Don W. Korte, Jr. LTC, MS 14 Jun 94
LTC Don W. Korte, Jr., Ph.D. Date
USAMRICD COR

Project Number G1555-9001 (8846)

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LTC Korte
R Menton
F Reid
QA
C Olson/File

Date June 17, 1994

To Study File SC930216, Protocol 98

From FM Reid

Subject Phase VIII Dosing and Modification

1. Progress To Date: On June 15, 1994, animal HFYBJC was dosed with WR269410 (PAHP) at 5.0 mg/kg and animal HFYAGL was dosed with the vehicle control. Blood samples were taken for hemoglobin and methemoglobin analysis, beginning approximately 1.0 hr after dosing. The 1.5 hr blood sample indicated that the PAHP-dosed animal was within the CN challenge range of 3.0 - 7.0 percent methemoglobin (5.1 percent). HFYBJC was prepared for CN challenge and the -1 min blood sample taken. The MHb level was 5.3 percent when CN challenge began. The second dog was infused with CN approximately 3.25 hr after dosing the vehicle control. On June 16, 1994, animal HFZBAV was dosed with WR269410 (PAHP) at 5.0 mg/kg and animal HFYAWJ was dosed with the vehicle control. Blood samples were taken for hemoglobin and methemoglobin analysis, beginning approximately 1.0 hr after dosing for the PAHP-dosed animal. The MHb level was at 2.7 percent methemoglobin. An additional blood sample (approximately 30 min latter) indicated that HFZBAV was 3.8 percent. A 2-hr sample indicated that the MHb level had dropped to 3.0 percent. The experiment was stopped. The Study Director consulted with LTC Korte concerning this week's dosing.

2. Consultation with LTC Korte: The four days of dosing Phase 8 were discussed. The following action was decided:

   a. Begin dosing the first two animals on Monday June 20, 1994 according to Protocol 98, Amendments and memos. The dosage of PAHP is 7.0 mg/kg in PEG 200. The dosage will be administered by capsule per os. The MHb level in the blood after pretreatment will be monitored at times suggested by the Study Director (based on data obtained during the previous week and Phase VII). When a MHb level of approximately 3 - 4 percent is reached, the animal may be prepared for CN challenge, in order to infuse CN while the blood MHb level is between 3.0 - 7.0 percent. The target MHb level is approximately 5 percent for CN infusion.

Approved By:

Frances M. Reid
Frances M. Reid, D.V.M., M.S.
Study Director

6-17-94
Date

Don W. Korte, Jr. LTC, MS
LTC Don W. Korte, Jr., Ph.D.
USAMRICD COR

17 Jun 94
Date

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Project Number G1555-9001 (8846)

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Date June 21, 1994

To File SC930216, Protocol 98

From Frances Reid

Subject Phase VI Multiple Dosing Blood Sampling Time Delayed

1. Blood Sampling Time Delay - On June 18, 1994, the 24 hr blood sample for animals HFZADP, HFZBAD, HFXAYK, and HFXBGH in Phase VI WR242511AE Multiple Dosing was delayed between 19 - 28 min (depending on the animal) from the original bleeding time.

Reason for Delay - The original blood samples drawn for these animals were run in the human mode. The technician recognized this error after the fourth animal, and set the instrument to the animal mode for the dog. The remaining two animals were sampled and analyzed for MHb and Hb in the dog mode on schedule. Additional blood samples were drawn from the first four animals and analyzed for MHb and Hb after completing the original sample collections. This resulted in time delays from the original blood collection of 28 min for HFZADP, of 25 min for HFZBAD, of 22 min for HFXAYK, and of 19 min for HFXBGH.

Corrective Action - The hemoximeter was set for the dog mode as soon as the technician discovered it was in the human mode. The remainder of the blood samples were drawn as scheduled and ran in the correct mode. The blood samples analyzed in the human mode were redrawn and reanalyzed in the correct mode. A memo to file was written documenting what occurred.

Impact on the Study - There are no adverse effects on the study caused by the delayed sample analysis. Compound WR242511AE is slow in producing MHb (approximately 0.6 percent MHb unit within a 12 hr period), and thus, an approximate half-hour delay is not expected to show change (within tenths) in the MHb level.

Approved By:

Frances M. Reid
Frances M. Reid, D.V.M., M.S.
Study Director

6-21-94
Date

Don W. Korte LTC, MS
LTC Don W. Korte, Jr., Ph.D.
USAMRICD COR

21 Jun 94
Date

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Internal Distribution
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Date June 28, 1994

To Task 92-28 Study File SC930216, Protocol 98

From Frances Reid

Subject Memorandum for the Record - Phase VI Multiple Dosing, Methodology, and Blood Sampling Times for WR269410 (PAHP)

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Phase VI: A Multiple Dose Pharmacodynamic and Pharmacokinetic Experiment with Multiple Doses of Compound WR269410.

WR269410

1. Test System - Animals used for Phase VI are those from Phases I and II. As with previous phases, a hematocrit and body weight are obtained within 48 hr of dosing. Feed is removed approximately 4 hours prior to dosing. Feed is returned approximately 2 hours after dosing.

2. Test Compound - WR269410 (PAHP) is administered every 8 hours, orally, in capsules similar to the method used in Phases VII and VIII of Task 92-28. The recommended dosage of WR269410 is 6 mg PAHP/kg in PEG 200 (based on FAX dated May 9, 1994 from Dr. Marino).

References to readministration of capsules, adjustments to schedules, dosing, dosing interval, and samples collected are found in Protocol 98, Addendum 1, Amendment 3, and this memo dated June 1994.

3. Blood Collection - Approximately 2 mL of heparinized blood is collected for hemoglobin (Hb), methemoglobin (MHb), and drug analyses. Additional blood samples may be taken based on results for MHb and Hb analyses as determined by the Study Director. If required, additional blood samples of approximately 0.5 mL may be collected for MHb and Hb analysis only. Blood samples are collected at the following times (plus or minus several minutes); A refers to sampling after the initial dose, B refers to blood sampling after the initial dose until the last sample time, C refers to blood sampling times after last dose the morning of Day 14, and 0 represents the blood sample around dosing.

A: In minutes -	0, 10, 20, 40, 60, 75, 90, 120, 180, 240, 360, 480.
B: In minutes -	0, 60 and 120 post-dosing. Additional MHb and Hb samples may be needed at approximately 150 and 180 min.
C: In minutes -	0, 10, 20, 40, 60, 75, 90, 120, 180, 240, 300, 360, 420, 480, 540, 600.

Blood samples for Hb and MHb measurement are analyzed on the Radiometer Hemoximeter as soon as possible after sample collection. The remainder of the sample is centrifuged and plasma collected.

4. WR269410 Analyses - The plasma samples collected are stored at approximately -70 degrees C until the end of this study (after the PAHP dosing and sample collecting are completed). The plasma samples are then shipped to Walter Reed Army Institute of Research or their designee for compound analysis.

5. PAHP Multiple Dose Experiment - The duration of the PAHP Multiple Dose Experiment will be two weeks.

6. Other Issues - The COR and Study Director will review the data (MHb only) on a timely basis and make any necessary adjustments to doses or times. Depending on results or evaluation by the Study Director of animals, the experiment may be terminated by the Study Director following consultation with the COR.

Approved By:

Frances M. Reid — 6-30-94
Frances M. Reid, D.V.M., M.S. — Date
Study Director

Don W. Korte, LTC, MS — 29 Jun 94
LTC Don W. Korte, Jr., Ph.D. — Date
USAMRICD COR

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Project Number G1555-9001 (8846)

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Date July 23, 1994

To Task 92-28 Study File SC930216, Protocol 98

From Frances Reid

Subject Memorandum for the Record - Phase VI Multiple Dosing for WR269410 (PAHP)

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Phase VI: A Multiple Dose Pharmacodynamic and Pharmacokinetic Experiment with Multiple Doses of Compound WR269410.

WR269410

Events occur on study that are not planned, and discussion of their possible impact on the results of the study are recorded periodically. Events noted during the initiation of Phase VI Multiple Dosing Experiment for WR269410 are as follows:

1. Review of the bleeding times established for WR269410 indicated a blood sample drawn at 480 minutes after the initial dosing and one at 0 minutes prior to next dosing. This required that two blood samples be drawn at the same time. The Study Director in consultation with the U.S. Army's Contracting Officer Representative (LTC Korte), decided to take a single blood sample to represent the 480 minute and the 0 minute sample.

2. Additional blood samples for hemoglobin and methemoglobin were taken at 3 and 4 hours post-dosing for 1 ~~3~~ dosing periods ~~s~~ after the initial dosing to determine when the approximate peak methemoglobin level occurred. The decision made was to proceed with the approximate 0, 60 and 120 minute blood samples.

1 FMR 7-25-94 ① FMR 7-25-94

Approved By:

Frances M Reid
Frances M. Reid, D.V.M., M.S.
Study Director

7-23-94
Date

Don W. Korte Jr LTC, MS
LTC Don W. Korte, Jr., Ph.D.
USAMRICD COR

25 Jul 94
Date

① ZE FMR 7-25-94

SC930216 Task 9228 Protocol 98

Date: July 26, 1994

To: Study File, Task 28, SC930216

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Subject: Memorandum for the Record - Phase V Methodology

Phase V: Fixed NaCN Challenge of Dogs Given a Methemoglobin Forming Compound.

1. Test Compound - WR302AG (PAPP) dissolved in PEG200 and administered IV.

2. Animals Used - Ten animals are randomly selected for Phase V from animals used in Phases III and IV of Task 9228.

3. Cyanide (CN) Analyses - Blood samples are analyzed for total cyanide levels only.

4. MHb Target Level - The level of methemoglobin (MHb) targeted in Phase V for WR302AG (PAPP) is 6.5%. A MHb range between 5% to 8% MHbemia, is established for CN infusion. Animals may be infused with CN once their measured MHb value falls within this range. Data from Phases II and III will assist in determining the dosage of PAPP to be administered. The time delay between dosing PAPP and CN challenge will vary. It is more important to challenge CN as close to 6.5% MHb as possible.

5. Washout Time Periods - A minimum of three work-weeks washout between previous phase and assignment to Phase V is required for each Phase V animal.

6. Blood Sampling - Approximately 1.5 mL of whole blood is collected for samples analyzed for Hb, MHb and total CN levels. Baseline blood samples prior to pretreatment may be taken during this phase to determine Hb and MHb levels only. Blood samples are collected, at a minimum, at -1 min, 0 min (Begin CN Infusion), and during CN infusion (similar to Phase III collections during CN infusion) for measurement of MHb, hemoglobin (Hb) values and total CN analyses. Additional blood samples for Hb and MHb only may be taken after PAPP administration and prior to CN infusion to determine MHb level for CN challenge. Additional blood samples may be collected for MHb and Hb only or to include total CN analysis if data indicates the necessity. Blood sampling timepoints post CN infusion may be the same as those utilized in Phase III. A schedule for blood sampling timepoints for each period is attached.

7. Method for Performing Phase V - A minimum of two animals per day may be infused with NaCN. Animals may be catheterized, anesthetized, intubated, instrumented, pretreated, infused with NaCN, and data recorded similar to Phase III method. Approximately a 4mg/mL NaCN dissolved in a saline solution (as previously prepared) will be infused at a rate approximately equal to 2 mL/min. A stock solution may be prepared in advance and analyzed for CN concentration prior to use. This concentration will be used to determine the two times the average time to respiratory arrest (2X AvTRA) fixed NaCN challenge as explained in Protocol 98 and Ammendment 5. The 2X AvTRA dose is infused, if an animal stops breathing prior to the infusion amount, the animal is treated (Hydroxylamine) as in previous phases when respiratory arrest occurs. If the fixed CN infused dose is given and respiratory arrest occurs after administration, then the animal is treated (Hydroxylamine) as in previous phases when respiratory arrest occurs. If respiratory Arrest does not occur (animal recovers), this will be noted on the data sheets and provided to the statistician.

Approved By:

Frances M. Reid — 7-26-94

Frances M. Reid, D.V.M., M.S.
Study Director

Date

Don W. Korte, Jr. LTC VAS — 26 Jul 94

LTC Don W. Korte, Jr., Ph.D.
USAMRICD COR

Date

SC930216 Task 9228 Protocol 98

BLOOD SAMPLING TIMES PHASE V

WR302AG

EVENT	BLOOD DRAW TIME (min)	AMOUNT (mL) Approximately	CHEM NOS
Administer WR302AG			
After WR302AG administration, blood samples (0.5 mL) may be taken for Hb and MHb analyses to determine time to start CN infusion.			
PreCN Infusion	- 1 prior to CN	1.5	
Pre CN Infusion	0	1.5	
Begin CN Infusion			
CN Infusion	1	1.5	
CN Infusion	2	1.5	
CN Infusion	3	1.5	
etc			
Continue 1 min, approximately 1.5 mL blood drawls until CN Infusion stopped			
Stop CN Infusion at 2X AvTRA	Begin blood w/drawl	1.5	
Administer Hydroxylamine 30 sec after Respiratory Arrest whenever it occurs			
Post CN Infusion	1	1.5	
Post CN Infusion	5	1.5	
Post CN Infusion	10	1.5	
Post CN Infusion	15	1.5	
Post CN Infusion	30	1.5	
Post CN Infusion	60	1.5	

Other samples as needed may be drawn.

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Date July 27, 1994

To Task 92-28 Study File SC930216, Protocol 98

From Frances Reid

Subject Memorandum for the Record - Termination of Phase VI, Multiple Dosing for WR269410 (PAHP)

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Phase VI: A Multiple Dose Pharmacodynamic and Pharmacokinetic Experiment with Multiple Doses of Compound WR269410.

WR269410

Events occur on study that are not planned, and discussion of their possible impact on the results of the study are recorded periodically. The Phase VI Multiple Dosing Experiment for WR269410 on Day 8 (dosing day 9) was terminated on July 27, 1994, following 24 hour blood samples (with analyses of hemoglobin and methemoglobin at a minimum). Phase VI was terminated for compound WR269410 to prevent further health effects in the animals on Phase VI.

A Phase VI animal on study collapsed on July 25, 1994. The animal had a low hematocrit, a low hemoglobin level, a high methemoglobin level, and intravascular hemolysis. These hematologic factors probably resulted in the collapse of the animal from hypoxia. The animal was removed from this study phase. The animal recovered without incident and thus far is doing well. The remaining animals were evaluated. An additional animal was removed from this phase because of an extremely low hematocrit (approximately 28 percent) and low hemoglobin (less than 8.0 grams percent). Mucous membranes were pale on all animals, but capillary refill time was within the normal range. Animals seemed depressed and were off feed in varying degrees. This information was presented to the Contracting Officer's Representative (COR, LTC Korte) with the recommendation that two of the animals be removed from further Phase VI testing, and that this phase be terminated. The COR agreed and requested that the last day's sample be done on the 4 remaining animals prior to terminating Phase VI. Since the health status of the remaining animals indicated this was possible, the Study Director agreed to perform the remaining blood sampling with the following changes:

1. Approximately 1 mL of blood would be drawn for each sample.

2. Some blood sampling times would be eliminated or sampling stopped if an animal's health status worsened and became a life-threatening situation.

The final dosing compound WR269410 was at 1600 hours on 7-26-94. Four animals were dosed and the blood collection schedule followed per the Study Director's instructions. To date, the animals are recovering without incident.

Approved By:

Frances M. Reid — 7-29-94
Frances M. Reid, D.V.M., M.S. — Date
Study Director

Don W. Korte, LTC, MS — 29 Jul 94
LTC Don W. Korte, Jr., Ph.D. — Date
USAMRICD COR

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Project Number G1555-9001 (8846)

Internal Distribution
LTC Stotts
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MREF/File

Date August 12, 1994

To Task 92-28 Study File SC930216, Protocol 98

From Frances Reid

Subject Memorandum for the Record - Validation of the Power Supply in PO-NE-MAH.

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Events occur on study that are not planned, and discussion of their possible impact on the results of the study are recorded periodically.

Phase V:

The PO-NE-MAH® system was turned on in preparation for beginning a new phase on Task 92-28 on Monday, August 8, 1994. The computer blew a 250 volt, 4 amp fuse in the power supply when turned-on. The fuse was replaced with a similar fuse (from another computer) and the system retried. This fuse was burned. PO-NE-MAH® company was contacted and the situation explained. They recommended disconnecting all accessories and then try to power-up. Additional fuses were purchased, that were similar but only 125 volts (the company said this was no problem). All accessories connected to the PO-NE-MAH® were disconnected and the fuse replaced. The fuse blew again. The PO-NE-MAH® company was called and the result discussed. The company suggested disconnect the A/D board and try. The burnt fuse was replaced and the A/D board disconnected. However, the fuse blew again. PO-NE-MAH® was consulted again. The remainder of the boards were removed and the fuse replaced. With just the power supply connected, the fuse blew. Thus, the power supply had a short in it. The PO-NE-MAH® company was consulted and they shipped a power supply Model #HS200A (Serial Number 79020511) to replace the old one. The computer was reassembled and powered-up. The computer booted past the password option and then produced the error message "HDD controller failure". The computer was disassembled again and the PO-NE-MAH® company (Scott Rogers) was called again. PO-NE-MAH® recommended that the hard drive connections were loose. One connection was found unattached and was re-attached. All connections were checked and secured. The computer was re-assembled and powered-up successfully. The PO-NE-MAH® system was re-evaluated for proper function. The power supply affects the hard drive, mother board, and floppy drive. The following procedures were done to access the performance of the new power Supply. The system was powered without any problems. The maim menu was evoked and various options accessed successfully. The hard drive was evaluated by accessing the AP software, access various menus (viewed on screen), retrieve files and data from the hard drive and optical disc drive, and exit the program. These were accomplished satisfactorily. To evaluate the mother board the AP software was accessed, transfer of data to and from the optical disc drive, and receipt of signals (data collection) was accomplished satisfactorily. A saved data file from the optical disc was retrieved and played for approximately 2 minutes, the system was functioning as expected (replay of data, etc). To evaluate the floppy drive, a file was retrieved from a floppy disc and a file was saved to a floppy disc. The floppy drive functioned as expected. On Monday, August 8, 1994, prior to beginning the next phase of Task 92-28, data was collected from a study animal and analyzed for heart rate and respiratory rate. The system produced data as expected and triggered appropriately for data collection to continue.

September 27, 1994
Phase VI Multiple Dosing Blood Sampling Time
Page 2

On August 8, 1994, data collection for heart rate transmitted to the PO-NE-MAH® system was lost after 22 minutes of data collection. The Astro-Med® recorder continued to collect the heart rate data This indicated a loose cable connection to the PO-NE-MAH® system for heart rate data transfer. The PO-NE-MAH® company was contacted and they recommended that a loose connection may be the problem. The study was completed and the cable connections on the back of the amplifiers, computer and transducers were checked. A loose connection was tightened and heart rate readings were transferred to the PO-NE-MAH® system again. The technician connected the leads to herself and collected heart rate data. The system collected heart rate data as expected. To date the system is functioning normally.

Approved By:

Frances M. Reid
Frances M. Reid, D.V.M., M.S.

8-12-94
Date

Study Director

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Project Number G1555-9001 (8846)

Internal Distribution
LTC Stotts
R Menton
F Reid
QA
MREF/File

Date September 28, 1994

To Task 92-28 Study File SC930216, Protocol 98

From Frances Reid

Subject Memorandum for the Record - In-Life Completion of Task 92-28 and Disposition of the Dogs.

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

1. In-Life Completion of Task 92-28.

   On 21 September 1994, Dr. Frances M. Reid of Battelle Memorial Institute received a letter that released the beagle dogs assigned to MREF Task 92-28 from this task. The following list of dogs have been released from the task:

ANIMALS USED ON TASK 92-28

1. HFXAYH - Phase I, Phase VI
2. HFZADI - Phase I, Phase VI
3. HFXAYK - Phase I, Phase VI
4. HFZADP - Phase I, Phase VI
5. HFZAYM - Phase II, Phase VI, Phase VII
6. HFXBDB - Phase II - died early in Phase II and replaced.
7. HFXBGH - Phase II - replaced HFXBDB, Phase VI
8. HFXBAD - Phase II, Phase VI
9. HFXAGU - Phase II, Phase VI, Phase VII, Pilot Phase VIII.
10. HFZBFI - Phase II, Phase VI, Phase VII
11. HFYBEI - Phase II, Phase VI, Phase VII, Pilot Phase VIII.
12. HFYBCP - Phase II, Phase VI, Phase VII
13. HFXBGI - Phase II, Phase VI, Phase VII
14. HFZADG - Phase III, Phase V.
15. HFZADD - Phase III, Phase V.
16. HFYAZF - Phase III,
17. HFYAIH - Phase III,
18. HFZBDC - Phase III, Phase V.
19. HFXAEN - Phase III, Phase V, Phase VII Pilot.
20. HFYBKC - Phase III, Phase V.
21. HFYBFM - Phase III, Phase V, Phase VII Pilot.
22. HFYAGL - Phase IV, Phase VIII

23. HFZANH - Phase IV, Phase VIII
24. HFYAWJ - Phase IV, Phase VIII, Phase V.
25. HFZAGX - Phase IV, Phase VIII, Phase V.
26. HFYAJM - Phase IV, Phase VIII, Phase V.
27. HFZAHB - Phase IV, Phase VIII
28. HFYATS - Phase IV, Phase VIII
29. HFYBJC - Phase IV, Phase VIII
30. HFZBAV - Phase IV, Phase VIII, Phase V.
31. HFZAAD
32. HFZAYK
33. HFYAWM
34. HFXBER
35. HFZAHI
36. HFZAVG

2. Disposition of the Dogs:

   a. The following animals are donated to Dr. Tim Sullivan:

      HFZAHI, HFZAVG, and HFZAYK.

   b. The following animals are donated to Ohio State University, College of Veterinary Medicine:

      HFXAYH, HFZADI, HFXAYK, HFZADP, HFZAYM, HFXBGH, HFXBAD, HFXAGU, HFZBFI, HFYBEI, HFYBCP, HFXBGI, HFZADG, HFZADD, HFYAZF, HFYAIH, HFZBDC, HFXAEN, HFYBKC, HFYBFM, HFYAGL, HFZANH, HFYAWJ, HFZAGX, HFYAJM, HFZAHB, HFYATS, HFYBJC, HFZBAV, HFZAAD, HFYAWM, and HFXBER.

      These animals are to be shipped on September 29, 1994.

Approved By:

Frances M. Reid — 9-28-94

Frances M. Reid, D.V.M., M.S.
Study Director

Date

Richard R Stotts — 28 SEP 94

LTC Richard R. Stotts, DVM, VC
USAMRICD COR

Date

Date: May 22,1995

To: Task 92-28 Study File, SC 930216

From: Kandy K. Audet / Kandy K. Audet

Subject: Memorandum for the record

Characterization of the Anticyanide Effect of Methemoglobinemia Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model.

The spreadsheets in the study files for phases III, IV, V and VIII have a statement at the top of the page that state; this information is only a draft and has not been reviewed by the Quality Assurance Unit. Spreadsheet drafts were put in the file because the sponsor requested information prior to a Quality Assurance review. These spreadheets were reviewed by the Quality Assurance Unit at a later date and contained the corrections made during the audit. However, the statement was not removed from the header when the corrections were printed.

Frances Mae Reid / FMR 5-24-95

Study Director.

Project Number G1555-9001 (8846)

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Internal Distribution

FM Reid
JB Johnson/File

Date January 31, 1995

To Task 92-28 Study File, SC930216

From Frances Reid

Subject Memorandum for the Record

Characterization of the Anticyanide Effect of Methemoglobinemia
Induced by Candidate Pretreatment Drugs in an Anesthetized Animal Model

Water and feed analysis records for this study, SC930216, are maintained with those of Battelle's Animal Facility at 505 King Avenue, Columbus, Ohio.

Approved By:

Frances M. Reid
Frances M. Reid, D.V.M., M.S.
Study Director

1-31-95
Date

APPENDIX C

TASK 92-28 CHEMISTRY REPORT

C-1.0 INTRODUCTION

The Task 92-28 Chemistry Report summarizes the results and methodology for the confirmation analyses. The report is divided into three major sections: (C.1) cyanide method validation and infusion solution concentration confirmation, (C.2) dose confirmation analysis of PAPP and PAHP, (C.3) dose analysis of hydroxylamine and (C.4) solution preparation sheet for 4.0 mg/mL NaCN infusion solution, 50.0 mg/mL hydroxylamine hydrochloride solution, 10.0 mg/mL methylene blue solutions, 250 mg/mL sodium thiosulfate solution, and 5.0 mg/mL atropine sulfate solution.

C-1.1. Cyanide

The following section of the report summarizes the chemistry results of the validation process for the automated microdistillation cyanide assay and the results of the analysis of the cyanide dosing samples assayed for MREF Task 92-28. The automated manifold configuration can be modified to measure either plasma free cyanide or total cyanide in a sample matrix. Both procedures were validated and the results are reported within this section. In addition, the results of the dose confirmation samples assayed using the total cyanide process have been summarized.

C-1.2. Methods

Both of the cyanide analyses utilize the Technician AutoAnalyzer® II continuous flow technology with a specialized manifold system. These procedures are described in Battelle SOP Number: MREF III-024-01 (See Attachment C-II). They incorporate dialysis, distillation, and the production of a fluorescent chelate which is measured by fluorescent emission.

C-1.2.1. Method Validation

The free cyanide procedure was validated using a 12 inch dialyzer over a working concentration range of 0.002 to 0.11 mM cyanide. The total cyanide procedure was validated

using a 6 inch dialyzer over a working concentration range of 0.04 to 0.11 mM. This required the use of two different control concentrations for each assay. The validation process was conducted on five days with standards being prepared from neat sodium cyanide daily.

C-1.2.2. Dose Confirmation Analysis

The dosing solutions were prepared on each day of experimentation. The analysis was performed using the total cyanide manifold configuration.

C-1.3.1. Validation Results

Table C-1. summarizes the results for the validation study.

TABLE C-1. VALIDATION RESULTS FOR CN^- ANALYSIS

			Control (mM) [a]			Dose [b]	
Date	Assay	$(R)^2$	Average Measured Value	STD	Percent RSD	Conc. [c] (mg/mL)	Percent Exposure [d]
9/17/93	Free CN^-	0.999	0.064	0.002	3.50		
			0.107	0.002	2.32		
9/17/93	Total CN^-	0.996	0.230	0.004	1.73		
			0.424	0.016	3.85		
						3.79	92.9
9/20/93	Free CN^-	0.999	0.058	0.002	3.23		
			0.103	0.012	12.0		
9/21/93	Total CN^-	0.999	0.226	0.006	2.50		
			0.403	0.007	1.73		
						3.90	95.7
9/22/93	Free CN^-	0.989	0.058	0.001	1.30		
			0.102	0.002	1.53		
9/22/93	Total CN^-	0.998	0.217	0.005	2.10		

Table C-1.
(Continued)

			Control (mM) [a]			Dose [b]	
Date	Assay	$(R)^2$	Average Measured Value	STD	Percent RSD	Conc. [c] (mg/mL)	Percent Exposure [d]
			0.387	0.010	2.46		
						3.72	91.2
9/23/93	Free CN^-	0.998	0.059	0.002	3.21		
			0.100	0.001	1.32		
	Total CN^-	0.998	0.220	0.002	0.85		
			0.407	0.011	2.77		
						4.02	98.5
9/27/93	Free CN^-	0.996	0.057	0.001	2.56		
			0.099	0.002	2.42		
	Total CN^-	0.988	0.213	0.009	4.07		
			0.377	0.011	2.99		
						**	

[a] Prepared concentration: 0.060, 0.100 for free CN^-, and 0.220, and 0.400 mM for total CN^-.
[b] Prepared concentration: 4.08 mg/mL, diluted 1/500 for analysis.
[c] mg/mL after converting from mM and correcting for the 1/500 dilution.
[d] Percent of expected.
** Error in sample preparation-concentration data not considered.

C-1.3.2. Dose Confirmation

The results for cyanide dose confirmation analyses are summarized in Figure C-1. and the average concentration value for each analysis day is given in Table C-2. along with a running average and standard deviation.

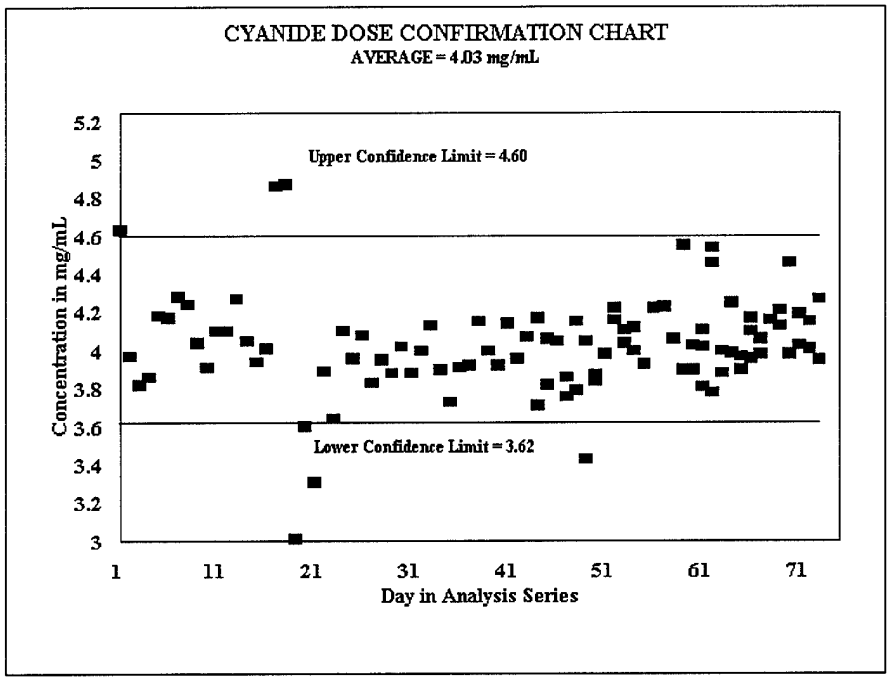


FIGURE C-1. CYANIDE DOSE CONFIRMATION DATA FOR DAYS 1 TO 80.

TABLE C-2. CYANIDE DOSE RESULTS

Analysis Day	Value (mg/mL)	Analysis Day	Value (mg/mL)
1	4.63	28	3.64
2	3.97	29	4.10
3	3.82	30	3.96
4	3.86	31	4.08
5	4.18	32	3.83
6	4.17	33	3.88
7	4.28	34	3.95
8	4.24	35	4.02
9	4.04	36	3.88
10	3.91	37	4.00
11	4.10	38	4.13
12	4.10	39	3.90
13	4.27	40	3.73
14	3.97	41	3.91
15	4.20	42	3.92
16	4.05	43	4.15
17	3.94	44	4.00
18	4.01	45	3.92
19	4.86	46	4.14
20	4.87	47	3.96
21	3.55	48	4.07
22	3.01	49	3.71
23	3.60	49	4.17
24	3.63	50	4.06
25	3.97	50	3.82
26	3.31	51	3.72
27	3.89	51	3.76

Table C-2.
(Continued)

Analysis Day	Value (mg/mL)	Analysis Day	Value (mg/mL)
52	4.02	68	4.02
52	3.95	69	3.78
53	4.05	69	4.48*
54	3.68	69	4.56
54	3.79	70	3.88
55	3.79	70	4.00
55	4.15	71	3.99
56	3.43	71	4.25
56	4.05	72	3.97
57	3.87	72	3.90
57	3.84	73	3.96
58	3.98	73	4.10
59	4.53	73	4.17
59	4.22	74	3.98
60	4.11	74	4.06
60	4.04	75	4.16*
61	4.00	76	4.21
61	4.12	76	4.13
62	3.93	77	4.46
63	4.22	77	3.98
64	4.23	78	4.19
65	4.06*	78	4.03
66	4.55*	79	4.15
66	3.90*	79	4.01
67	3.90	80	4.27
67	4.03	80	3.95
68	4.11	Average Standard Deviation	4.02mg/mL 0.268
68	3.81		

* Data not used.

C-1.3.3. Control Samples

The control solutions used for validation were also analyzed on each dosing day. The average values for each day are shown at each concentration (Figure C-2. through Figure C-5.).

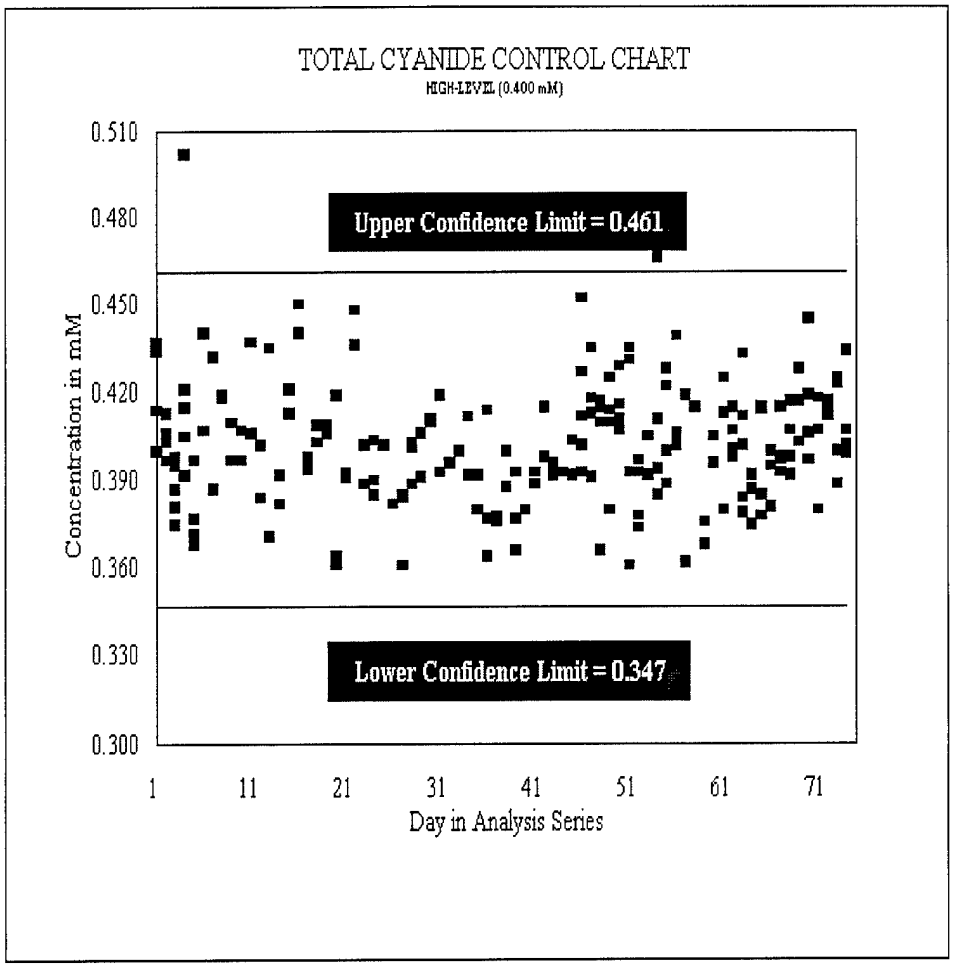


FIGURE C-2. TOTAL CYANIDE CONTROL 0.400 mM

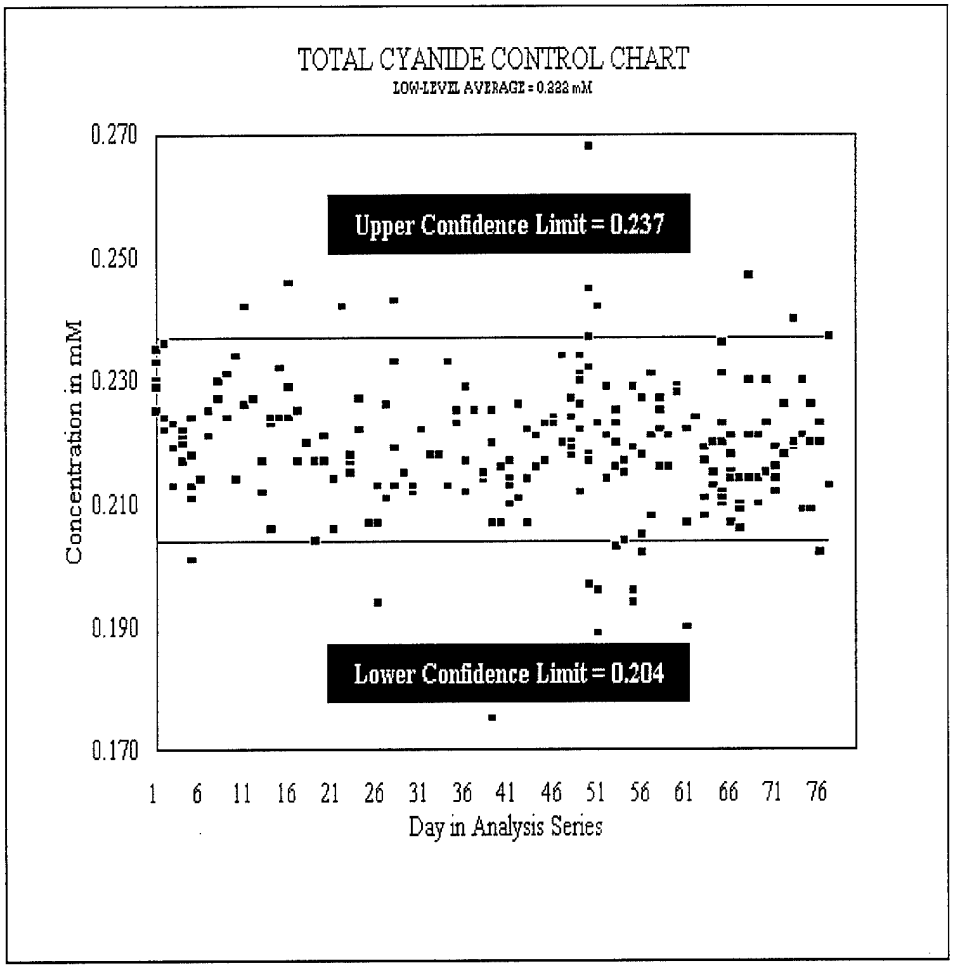


FIGURE C-3. TOTAL CYANIDE CONTROL 0.220 mM

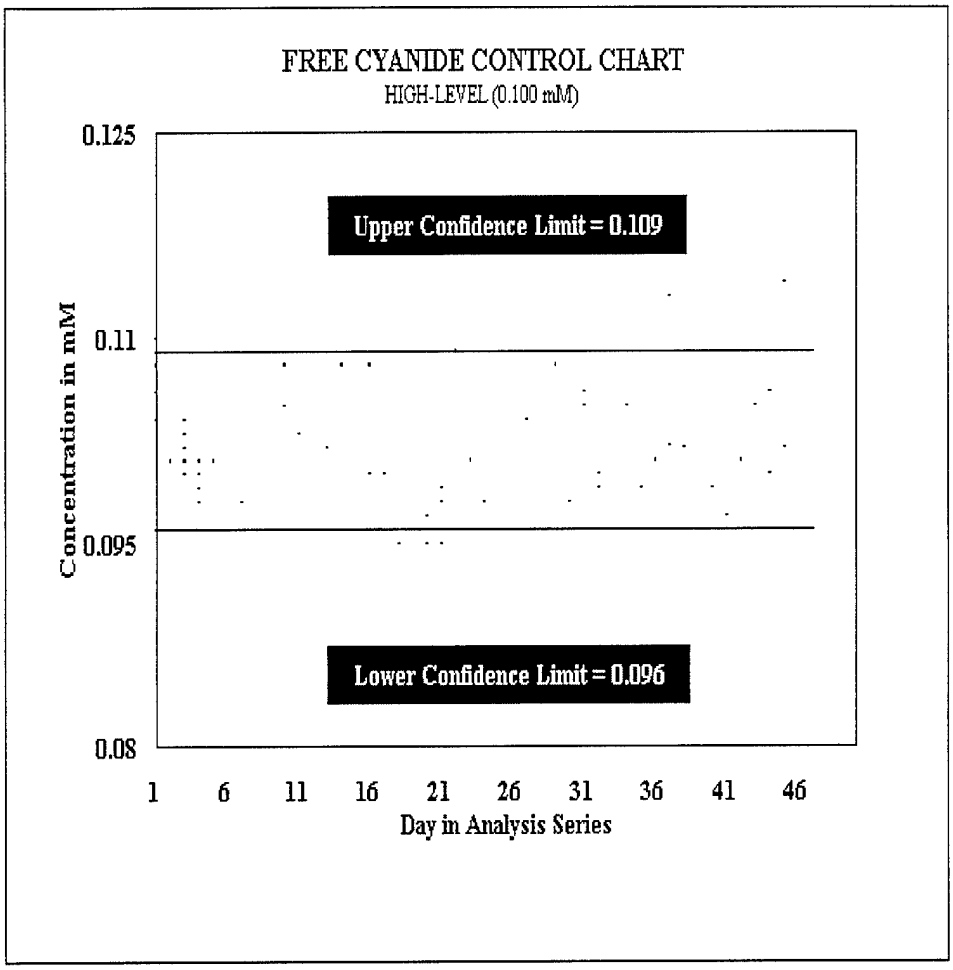


FIGURE C-4. FREE CYANIDE CONTROL 0.100 mM

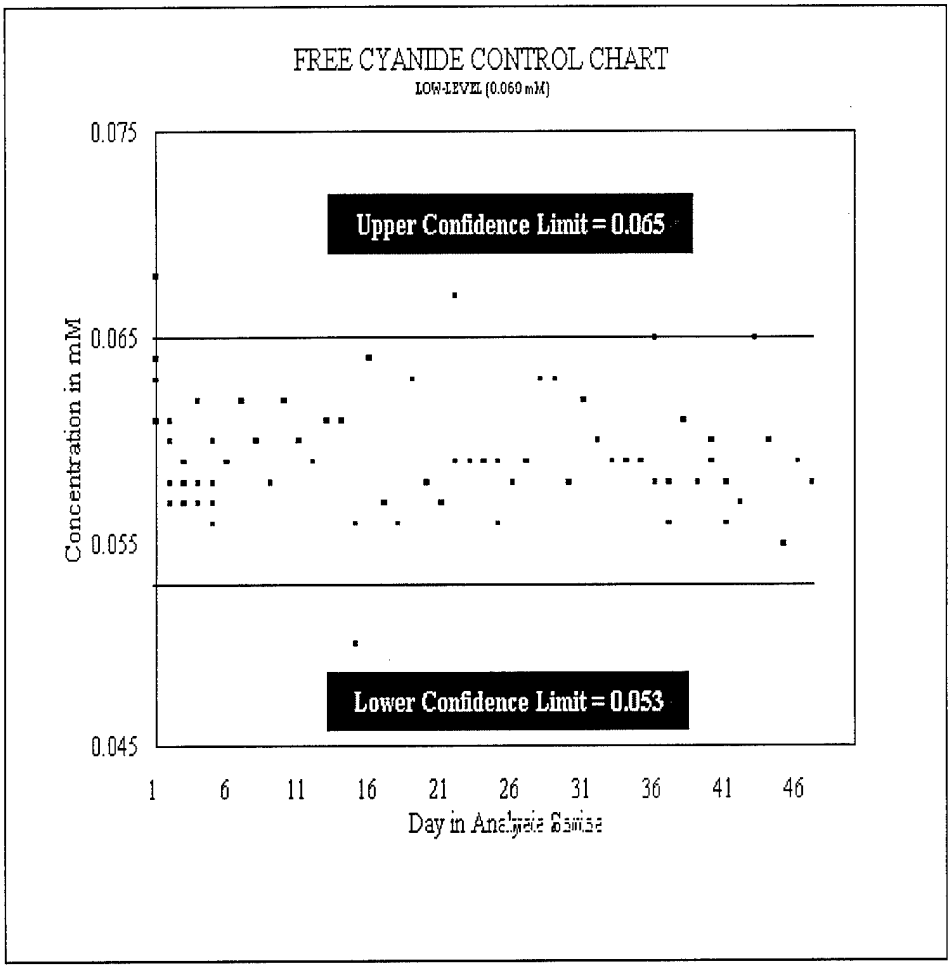


FIGURE C-5. FREE CYANIDE CONTROL 0.060 mM

C-1.4. Discussion

From the results in Table C-1., the average values (shown with standard deviation as ±) over the five day validation period are: 0.059 ± 0.003 mM, 0.102 ± 0.003 mM, 0.221 ± 0.007 mM, 0.400 ± 0.018 mM, and 3.86 ± 0.13 mg/mL. The relative standard deviation for the average values is 5.1 percent, 2.9 percent, 3.2 percent, 4.5 percent, and 3.4 percent, respectively, and the percent error for each value is 1.7 percent, 2.0 percent, 0.5 percent, 0.0 percent, and 5.4 percent, respectively.

The average and standard deviation for each of the four control samples analyzed over the 73 day test period are: 0.060 ± 0.003 mM, 0.102 ± 0.005 mM, 0.219 ± 0.016 mM, and 0.403 ± 0.021 mM. The relative precision for each control sample is 5.0, 4.9, 7.3, and 5.2 percent, respectively. The relative accuracy (expressed as percent error) for these samples is 0.0, 2.0, 0.5, and 0.8 percent, respectively. Consequently, the dose solution concentrations reported in Table C-2. should represent the dose concentration well below the ten percent error level.

C-2.0 DOSE ANALYSIS OF PAPP AND PAHP

C-2.1. Introduction

Both the p-aminopropiophenone (PAPP) and the p-aminoheptanophenone (PAHP) were analyzed by HPLC with spectrophotometric detection at 270 nm. The analysis method, Chemistry Method No. 12, is given in Attachment C-III.

C-2.2. Results

The analysis results for the confirmation of PAPP and PAHP dose concentrations are summarized in Tables C-3. and C-4., respectively. Measured concentrations reflect the average of triplicate dilutions of the dosing solution.

TABLE C-3. PAPP DOSE ANALYSIS RESULTS

Analysis Date	Nominal Concentration (mg/mL)	Measured Concentration (mg/mL)
9/29/93	1.01	0.96
9/30/93	4.04	3.89
10/6/93	3.00	2.93
10/7/93	4.01	4.07
10/13/93	4.01	3.88
10/14/93	2.00	1.85
10/20/93	6.03	6.23
10/21/93	3.04	2.97
10/27/93	5.04	4.61
11/3/93	2.03	1.94
1/19/94	2.03	2.04
1/20/94	2.02	1.98
1/21/94	2.00	2.09
1/25/94	1.98	1.93
1/26/94	2.98	2.94
1/27/94	4.99	5.23
1/31/94	2.00	1.99
2/1/94	3.00	2.82
2/2/94	5.05	5.20
2/7/94	2.02	2.02
2/8/94	2.01	1.95
2/9/94	5.00	5.06
2/16/94	2.00	2.03
2/17/94	3.01	3.03
2/21/94	6.01	6.15
2/23/94	2.00	2.01

Table C-3.
(Continued)

Analysis Date	Nominal Concentration (mg/mL)	Measured Concentration (mg/mL)
2/24/94	3.00	3.01
2/28/94	3.01	3.10
3/1/94	7.00	7.01
3/3/94	2.02	2.04
3/7/94	3.03	3.08
3/8/94	4.01	3.84
3/10/94	2.00	2.00
4/11/94	4.02	4.05
4/12/94	4.02	3.93
4/13/94	3.00	3.05
4/14/94	3.00	3.03
4/15/94	3.00	3.06
8/8/94	5.00	5.25
8/9/94	5.00	5.27
8/10/94	4.99	5.20
8/11/94	6.04	6.25
8/12/94	4.04	4.23

TABLE C-4. PAHP DOSE ANALYSIS RESULTS

Analysis Date	Nominal Concentration (mg/mL)	Measured Concentration (mg/mL)
5/16/94	121	119
5/20/94	120	133

Table C-4.
(Continued)

Analysis Date	Nominal Concentration (mg/mL)	Measured Concentration (mg/mL)
5/26/95	121	136
5/27/94	121	133
6/10/94	120	128
6/16/94	120	6[(1)]
6/17/94	120	145
7/6/94	120	147
7/15/94	124	131
7/20/94	124	143

[(1)] Data not used. Internal standard addition error. Sample rediluted and reanalyzed on 6/17/94.

C-2.3. Conclusions

Analyses of the PAPP dosing solutions indicated that all solution concentrations were within 10 percent of the nominal values. Analysis of the PAHP dosing solutions indicated that all concentrations were within 10 percent of the nominal values with the exception of the 5/26/96, 6/16/96, 6/17/96, and 7/6/96 analyses.

C-3.0 DOSE ANALYSIS OF HYDROXYLAMINE

C-3.1. Introduction

Hydroxylamine was prepared in ultrapure water at a nominal concentration of 50 mg/mL. Fifty-mL aliquots of the solution were sealed in clear glass ampules and stored at room temperature. The working solution was analyzed each day of use according to Chemistry Method No. 13, provided in Attachment C-IV.

C-3.2. Results

The analysis results for the confirmation of hydroxylamine dose concentrations are summarized in Table C-5. Measured concentrations reflect the average of triplicate titrations of the dosing solution.

TABLE C-5. HYDROXYLAMINE DOSE ANALYSIS RESULTS

Analysis Date	Hydroxylamine Dose Measured Concentration (mg/mL)	Hydroxylamine Standard Measured Concentration (mg/mL)
11/29/93	9.6	49.6
11/30/93	50.2	49.9
12/3/93	51.0	49.7
12/6/93	48.8	49.7
12/7/93	49.9	49.0
12/8/93	50.0	48.8
12/9/93	49.6	48.5
12/13/93	50.0	48.9
12/14/93	49.6	49.3
12/15/93	49.6	48.9
12/16/93	50.1	49.5
12/20/93	49.6	48.8
12/21/93	49.6	49.0
12/22/93	50.2	49.8
12/23/93	49.4	49.3
12/29/93	49.6	49.4
12/30/93	49.6	49.4
1/18/94	49.9	50.1
1/19/94	50.5	49.6

Table C-5.
(Continued)

Analysis Date	Hydroxylamine Dose Measured Concentration (mg/mL)	Hydroxylamine Standard Measured Concentration (mg/mL)
1/20/94	50.5	50.6
1/21/94	49.5	49.3
1/21/94	49.6	49.3
1/25/94	48.8	49.4
1/26/94	48.8	49.5
1/27/94	48.0	49.2
1/31/94	49.4	49.6
2/1/94	49.0	50.0
2/2/94	49.9	49.3
2/3/94	48.5	48.8
2/7/94	49.7	50.0
2/8/94	48.5	48.7
2/9/94	50.3	50.5
2/10/94	50.2	49.5
2/14/94	48.9	48.8
2/15/94	48.8	48.8
2/16/94	49.0	49.7
2/17/94	48.8	49.0
2/21/94	48.7	49.0
2/22/94	48.5	48.1
2/23/94	49.0	48.5
2/24/94	49.3	49.5
2/25/94	50.4	49.9[1]
2/28/94	49.0	48.8

Table C-5.
(Continued)

Analysis Date	Hydroxylamine Dose Measured Concentration (mg/mL)	Hydroxylamine Standard Measured Concentration (mg/mL)
3/1/94	49.7	49.5
3/2/94	49.9	50.4
3/3/94	49.1	49.3
3/7/94	49.9	49.7
3/8/94	49.3	49.8
3/9/94	49.3	49.8
3/10/94	49.6	49.8
4/1/94	48.5	49.0
4/4/94	49.3	48.8
4/5/94	49.9	49.7
4/6/94	48.7	49.0
4/7/94	49.1	48.4
4/8/94	49.0	48.8
4/11/94	48.8	48.6
4/12/94	49.0	49.8
4/13/94	49.0	48.9
4/14/94	45.3	48.6
4/15/94	49.2	49.7
4/25/94	49.5	49.2
4/26/94	49.3	49.6
4/27/94	49.9	50.3
4/28/94	49.3	49.7
4/29/94	49.8	49.8
5/5/94	49.1	50.1

Table C-5.
(Continued)

Analysis Date	Hydroxylamine Dose Measured Concentration (mg/mL)	Hydroxylamine Standard Measured Concentration (mg/mL)
6/9/94	48.5	49.1
6/13/94	48.8	49.0
6/15/94	48.2	47.9
6/20/94	49.5	49.4
6/22/94	49.3	49.3
6/23/94	49.0	48.9
6/24/94	49.1	48.4
6/27/94	50.6	49.8
6/29/94	49.7	48.8
6/30/94	48.8	48.2
7/1/94	48.5	48.8
8/8/94	49.6	49.3
8/9/94	49.6	48.9
8/10/94	49.2	49.0
8/11/94	49.0	49.6
8/12/94	50.3	49.9

[1] Result reflects sample reanalysis. Original aliquot contaminated.

C-3.2. Conclusions

Analysis of the hydroxylamine dosing solution indicated that all solution concentrations were within 10 percent of the nominal values with the exception of the 11/29/93 analysis.

ATTACHMENT C-I.

SOLUTION PREPARATIONS

Task 28 Study #SC930216
Solution Preparation

1) 4.0 mg/mL Sodium Cyanide Solution: Weigh 100.0 mg up to 100.5 mg of NaCN salt into a sterile 25 mL volumetric flask in ventilated balance enclosure. Place glass stopper in flask, wrap with Parafilm®, and then transport flask to approved fume hood. Fill the flask approximately 2/3 full with sterile saline, stopper, and mix by inversion until the salt is completely solubilized. Once the salt is dissolved, the solution is brought to the appropriate volume mark and again mixed for approximately 20 seconds. Attach a sterile Acrodisc® syringe filter (0.2 μm, Lot #8216) to a 30 cc sterile disposable plastic syringe. Remove the syringe plunger and pour the NaCN solution into the syringe, replace the plunger and expel the NaCN solution into a sterile 100 mL serum vial. Cap the serum vial. Use a 1 mL disposable plastic syringe and sterile needle to remove a 1.0 mL sample. Place 0.9 mL of the solution sample in a sterile 1 mL serum vial, cap, and save for analysis by chemistry. Expel the remaining 0.1 ml solution sample onto a pH indicator strip to verify the pH > 7. Record the pH on the solution preparation worksheet.(Expiration date 1 day)

2) 50.0 mg/mL Hydroxylamine Hydrochloride Solution: Weigh 25.0000 g up to 25.0005 g of HA-HCl in ventilated balance enclosure on weigh paper. Quantitatively transfer the weighed material into a sterile 500 mL volumetric flask. Place glass stopper in flask, wrap with Parafilm®, and then transport flask to approved fume hood. Fill the flask approximately 2/3 full with sterile saline, stopper, and vortex until Hydroxylamine Hydrochloride is completely solubilized. The solution is brought to the appropriate volume mark and again vortexed for approximately 10 seconds. Attach a sterile Acrodisc® syringe filter (0.2 μm, Lot #8216) to a 30 cc sterile disposable plastic syringe. Remove the syringe plunger and pour 25.0 mL of the HA-HCl solution into the syringe, replace the plunger and expel the HA-HCl solution into a sterile 30 mL amber serum vial. Cap the serum vial. Send this sample to chemistry for verification of concentration (concentration tolerance range 47.5 mg/mL to 52.5 mg/mL). If sample is within tolerance range then repeat filtration and aliquot procedure with new Acrodisc® syringe filter (0.2 μm, Lot #6369), until stock solution is exhausted. If sample is out of tolerance range then repeat solution preparation until acceptable concentration is achieved. (Expiration date 1 year, daily concentration verification)

3) 10.0 mg/mL Methylene Blue Solution: Weigh 500.0 mg up to 500.5 mg of Methylene Blue in ventilated balance enclosure on weigh paper. Quantitatively transfer the weighed material into a sterile 50 mL volumetric flask. Place glass stopper in flask, wrap with Parafilm®, and then transport flask to approved fume hood. In a small beaker heat approximately 60.0 mL of sterile saline to a temperature warm to the touch. Fill the flask approximately 2/3 full with the warm sterile saline, stopper, and vortex. Place flask in a beaker of (warm to the touch) water in sonicator until Methylene Blue is completely solubilized. The solution is brought to the appropriate volume mark with room temperature sterile saline and again vortexed for approximately 10 seconds. Attach a sterile Acrodisc® syringe filter (0.2 μm, Lot #8216) to a 60 cc sterile disposable plastic syringe. Remove the syringe plunger and pour the Methylene Blue solution into the syringe, replace the plunger and expel 25.0 ml of the Methylene Blue solution into a sterile 30 mL serum vial. Cap the serum vial. Filter the remaining solution into a second 30 mL serum vial. Cap the serum vial. (Expiration date 30 days)

4) 250.0 mg/mL Sodium Thiosulfate Solution: Weigh 12.5000 g up to 12.5005 g of Na Thiosulfate in ventilated balance enclosure on weigh boat. Quantitatively transfer the weighed material into a sterile 50 mL volumetric flask. Place glass stopper in flask, wrap with Parafilm®, and then transport flask to approved fume hood. Fill the flask approximately 2/3 full with sterile saline, stopper, and vortex until Na Thiosulfate is completely solubilized. The solution is brought to the appropriate volume mark and again vortexed for approximately 10 seconds. Attach a sterile Acrodisc® syringe filter (0.2 μm, Lot #8216) to a 30 cc sterile disposable plastic syringe. Remove the syringe plunger and pour 25.0 mL of the Na Thiosulfate solution into the syringe, replace the plunger and expel the Na Thiosulfate solution into a sterile 30 mL amber serum vial. Cap the serum vial. Replace the Acrodisc® syringe filter (0.2 μm, Lot #6369) and filter the remaining solution into a second 30 mL amber serum vial. Cap the serum vial. (Expiration date 7 days)

5) 5.0 mg/mL Atropine SO_4 Solution: Weigh 25.0 mg up to 25.5 mg of Atropine SO_4 in a ventilated balance enclosure on weigh paper. Quantitatively transfer the weighed material into a sterile 25 mL volumetric flask. Place glass stopper in flask, wrap with Parafilm®, and then transport flask to approved fume hood. Fill the flask approximately 2/3 full with sterile saline, stopper, and vortex until Atropine SO_4 is completely solubilized. The solution is brought to the appropriate volume mark and again vortexed for approximately 10 seconds. Attach a sterile Acrodisc® syringe filter (0.2 μm, Lot #8216) to a 30 cc sterile disposable plastic syringe. Remove the syringe plunger and pour the Atropine SO_4 solution into the syringe, replace the plunger and expel the Atropine SO_4 solution into a sterile 30 mL amber serum vial. Cap the serum vial. (Expiration date 6 months)

ATTACHMENT C-II.

STANDARD OPERATING PROCEDURE FOR THE MEASUREMENT OF PLASMA FREE CYANIDE AND BLOOD TOTAL CYANIDE: A RAPID COMPLETELY AUTOMATED MICRODISTILLATION ASSAY.

BATTELLE COPY

Manual Number: QAU.01

Battelle SOP Number: MREF III-024-01

Key Words: Technicon®, Cyanide, Microdistillation

STANDARD OPERATING PROCEDURE (SOP) FOR THE MEASUREMENT OF PLASMA FREE CYANIDE AND BLOOD TOTAL CYANIDE: A RAPID COMPLETELY AUTOMATED MICRODISTILLATION ASSAY

Originated by: Timothy L. Murphy
Timothy L. Murphy, M.S., MT(ASCP) Date 12-3-93

Approved by: David W. Hobson
David W. Hobson, Ph.D., D.A.B.T.
Principal Investigator and Manager
Medical Research and Evaluation Facility Date 12/3/93

Approved by: David Stitel
CIH, Safety and Surety Officer Date 12/3/93

Reviewed and Registered by QAU: Kathleen E. Reed Effective Date 12-3-93

Distribution List:

Quality Assurance Unit
SOP Manual(s)

Battelle
505 King Avenue
Columbus, Ohio 43201-2693

I. Scope

This SOP describes the use of Technicon AutoAnalyzer® (AA) II modules for the direct measurement of both free cyanide (CN^-) in plasma and total CN^- in whole blood. Samples may be obtained for this assay using methods described in Battelle SOP MREF VII-020 or by using equivalent procedures.

II. Purpose

The purpose of this SOP is to detail the procedures for the automated measurement of plasma free CN^- and blood total CN^-. The method incorporates dialysis, distillation, absorption and the production of a fluorescent chelate. The distillation-absorption assembly, an extremely critical and unique all glass construction, is essential to the precise separation of CN^- vapor from liquid. No prior isolation technique is required and a readout is provided approximately 12 min after sampling. Sensitivity is adjustable to cover a broad range of CN^- concentrations from 1 to 4,000 μM/L. Both thiocyanate and thiosulfate, which interfere in the estimation of CN^- by many methods, do not affect the CN^- measurement at a concentration of approximately 1 mM.

III. References

Battelle SOP GEN VII-006, "Standard Operating Procedure for Handling and Storage of Highly Toxic Substances, Select Carcinogens, and Reproductive Toxins".

Battelle SOP MREF VII-020, "Standard Operating Procedure for Collecting Blood and Installing Catheters at the MREF".

Battelle SOP MREF IX-001, "Standard Operating Procedure for Use of Cyanide Salts/Solutions".

Facility Safety and Surety Plan (FSSP) SOP MREF-55, "Standard Operating Procedure for Segregation, Storage, and Disposal of Infectious Wastes at the MREF".

Groff, W.A., Stemler, F.W., Kaminskis, A., Froehlich, H.L., Johnson, R.P. "Plasma Free Cyanide and Blood Total Cyanide: A Rapid and Completely Automated Microdistillation Assay". Clin. Tox. 23:133-163, 1985.

Hanker, J.S., Gelberg, A., Witten, B. "Fluorometric and Colorimetric Estimation of Cyanide and Sulfide by Demasking Reactions of Palladium Chelates". Anal. Chem. 30:93-95, 1958.

Material Safety Data Sheets (MSDS) are available in the administrative area of the MREF or through Battelle's Safety Office, 505 King Avenue, Columbus, Ohio.

IV. Definitions

Technicon AutoAnalyzer® - This instrument, which is a product of Technicon Instrument Corp., is a continuous flow analytical system consisting of separate modules for sampling, reagent delivery, detection, and recording.

Cyanide - Cyanide is an extremely toxic compound which is rapidly absorbed into blood, freely crosses cell and mitochondrial membranes and arrests cellular respiration by inhibition of the terminal cytochrome oxidase (cytochrome a_3).

V. Procedures

A. Instrumentation

The analytical system consists of the following modules from Technicon Instruments Corporation, Tarrytown, NY.

1. Sampler IV.
2. Proportioning pump IV.
3. 3 dialyzers (6, 12 and 24 inch lengths) with type C membranes.
4. Research cartridge kit (Type A) with silicone oil heating bath at 115 $\pm$ 5 degrees C.
5. Fluorometer III with Corning Glass filters (excitation-#5970 with maximum transmission at 370 nm and emission-#4308 and #3389 with maximum transmission at 470 nm and sharp cut off below 400 nm).
6. Single pen recorder with percent transmittance chart paper.

B. Materials to be Used

Monobasic potassium phosphate (KH_2PO_4), sodium hydroxide (NaOH), Brij 35, Triton X-100, sulfuric acid (H_2SO_4), glycine ($C_2H_5NO_2$), sodium chloride (NaCl), 8-hydroxy-5-quinoline sulfonic acid ($C_9H_7NO_4S$), palladium chloride ($PdCl_2$), magnesium chloride ($MgCl_2$), sodium cyanide (NaCN), 4-dimethylaminophenol (4-DMAP), potassium carbonate (K_2CO_3), methanol (CH_3OH), diethyl ether $(CH_3CH_2)_2O$, millipore-grade water, silicone oil, and pH standards (4.0, 7.0, and 10.0).

The materials listed above should be of reagent grade or better quality. Unless specified, multiple suppliers are available for all materials.

C. Hazards Involved

Hazards involved are listed in Battelle SOPs MREF IX-001, GEN VII-006 and in the appropriate MSDS.

D. Equipment

The equipment used within this SOP includes those listed in Battelle SOP MREF IX-001 plus the following: refrigerator, volumetric flasks (10, 100, 1,000-mL), 1-L glass bottles, serological pipettes (1 and 5-mL), pipette bulbs, tissue paper, graduated cylinders (100 and 250-mL), sample racks, pH meter, centrifuge, vortex mixer, Eppendorf pipettor, pipettor tips, heat shrinkable tubing (5/32" ID), recorder pen, percent transmittance chart paper, Buchner funnel, and filter paper.

The following items are available from Bran + Luebbe, Inc. or Alpkem Corp.: mixing coils (part #157-B095-01), mixing coils (part #157-0248-01E), mixing coils (part #170-0103-01), heating bath coil (part #157-0224-01), air injection fitting (part #116-0492-01), sample injection fittings (part #116-0489-01), manifold trap (part #116-0110-01), glass tubing (part #116-0223-03), pump tube (part #116-0549P06), pump tube (part #116-0549P07), pump tube (part #116-0549P10), pump tube (part #116-0549P11), pump tube (part #116-0549P14), pump tube (part #116-0549P15), pump tube (part #116-0549P18), sampler probe (part #171-0658-01), pump end blocks (part #133-0122P01), side rails (part #133-0019-01), 0.5 mL sample cups (part #127-0090P01), air bar tubing (part #116-0543P01), transmission tubing (part #116-0528P01), sleeving (part #562-0005P01), T-connector (part #116-0202-03), T-connector (part# 116-0200-03), T-connector (part #116-0200P01), T-connector (part #116-0200P02), T-connector (part #116-0200P03), nipples (part #116-0002P01), nipples (part #116-0003P01), nipples (part #116-0004P01), nipples (part #116-0005P01), and nipples (part #116-0061P05).

E. Requirements

All personnel must be trained in the use of the Technicon AutoAnalyzer® per the Technicon Operation and Maintenance Manual.

F. Decontamination, Emergency Procedures, and First Aid Procedures

All operators are required to read, sign, and be familiar with Battelle SOPs GEN VII-006 and MREF IX-001.

G. Preparation of CN^- Assay Reagents

1. Chelate: potassium bis (5-sulfoxino) palladium (II). Add 4.50 g of 8-hydroxy-5-quinoline sulfonic acid to a solution of palladous chloride (2.14 g) in 300 mL of 5 percent sulfuric acid. Heat to boiling and then cool to room temperature. Add saturated potassium carbonate solution until the evolution of carbon dioxide ceases. The chelates separates as a fine, yellow precipitate. Collect the precipitate on a filter using a Buchner funnel and wash successively with 10 percent potassium carbonate solution, water, methanol, and diethyl ether. Allow the precipitate to dry in air. The estimated shelf life of this chemical is 5 years after the preparation date. Store at room temperature.

2. Free Cyanide Assay

   a. Phosphate buffer, pH 7.4, 0.05 M. Transfer 250.0 mL of 0.2 M acid potassium phosphate and 197.5 mL of 0.2 N sodium hydroxide to a 1 L volumetric flask. Dilute to 1 L with distilled water. The estimated shelf life of this solution is 6 months after the preparation date. Store at room temperature.

   b. First diluent: Phosphate buffer, pH 7.4, 0.05 M containing 1.0 mL Brij 35 per L. Prepare fresh each day. Store at room temperature.

   c. Second diluent: Phosphate buffer, pH 7.4, 0.05 M containing 1.0 mL Brij 35 per L. Prepare fresh each day. Store at room temperature.

   d. Recipient solution: Phosphate buffer, pH 7.4, 0.05 M containing 0.1 mL Brij 35 per L. Prepare fresh each day. Store at room temperature.

3. Total Cyanide Assay

   a. First diluent: Triton X-100, 0.5 percent. Add 5.0 mL of Triton X-100 to 1 L of distilled water. Prepare fresh each day. Store at room temperature.

   b. Second diluent: Sulfuric acid, 0.5 percent. Add 5.0 mL of concentrated sulfuric acid to approximately 500 mL of distilled water. Mix and dilute to 1 L with distilled water. The estimated shelf-life of this solution is six months after the date of preparation. Store at room temperature.

c. Stock recipient solution: Sulfuric acid, 0.25 percent. Add 2.5 mL of concentrated sulfuric acid to approximately 500 mL of distilled water. Mix and dilute to 1 L with distilled water. The estimated shelf-life of this solution is six months after the date of preparation. Store at room temperature.

d. Working recipient solution: Sulfuric acid, 0.25 percent, containing 0.1 mL Brij 35 per L. Prepare fresh each day. Store at room temperature.

4. Free and Total Cyanide Assays

a. Stock glycine-sodium chloride solution. Dissolve 77.4 g glycine and 58.6 g sodium chloride in distilled water. Dilute to 1 L. Refrigerate when not in use. The estimated shelf life of this solution is 6 months after the preparation date.

b. Glycine buffer, pH 10. Add 63.0 mL of stock glycine-sodium chloride solution to approximately 850 mL distilled water. Adjust to pH to 10.0 by addition of 1.0 N sodium hydroxide. Dilute to 1 L with distilled water. The estimated shelf life for this solution is one week after the date of preparation. Store at room temperature.

c. Stock chelate solution (chelate prepared in Section V.G.1). Potassium bis (5-sulfoxino) palladium (II). Dissolve 0.120 g chelate in 1 L distilled water. The estimated shelf life of this solution is six months after the preparation date. Store at room temperature.

d. Working chelate solution. Add 100 mL of stock chelate solution to approximately 500 mL distilled water. Dilute to 1 L with distilled water and add 1 mL Brij 35. The estimated shelf life for this solution is one week after the date of preparation. Store at room temperature.

e. Stock magnesium chloride solution. Dissolve 12.0 g magnesium chloride in 1 L distilled water. The estimated shelf life of this solution is six months after the preparation date. Store at room temperature.

f. Working magnesium chloride solution. Add 100 mL of stock magnesium chloride solution to approximately 500 mL distilled water. Dilute to 1 L with distilled water and add 1 mL Brij 35. The estimated shelf life for this solution is

one week after the date of preparation. Store at room temperature.

g. Isotonic saline. Dissolve 9.0 g sodium chloride in 1 L distilled water. The estimated shelf life of this solution is 6 months after the preparation date. Store at room temperature.

h. Stock CN standard, 0.04 M. Following the safety precautions and procedures stated in Battelle SOP MREF IX-001, dissolve 0.19604 g sodium cyanide and dilute to 100 mL with 0.1 N sodium hydroxide. Refrigerate when not in use. The estimated shelf life for this solution is one week after the date of preparation.

i. Working CN standards. Dilute appropriate volumes of stock CN standard with 0.01 N sodium hydroxide. Refrigerate when not in use. The estimated shelf life for this solution is one week after the date of preparation.

j. 4-Dimethylaminophenol (4-DMAP), 0.5 g/dL. Dissolve 0.05 g 4-DMAP in 10 mL distilled water. Refrigerate when not in use. The estimated shelf life for this solution is one week after the date of preparation.

H. Assay Procedure

1. The Technicon AutoAnalyzer® is operated following Technicon Operation and Maintenance Manuals. The flow diagram of the automated CN^- manifold is shown in the Attachment. Cups containing samples or standards are placed on the sample platter with the appropriate number of saline wash cups between the samples (2). The sampler is operated at a rate of 30/hour with a 120 second wash time resulting in an effective sampling rate of 10/hour. Before the start of analysis, the waste reservoir is filled approximately one-third full with a solution that has 5 percent available chlorine.

2. Instrument Start Up

   a. Pump water through manifold and isotonic saline to sampler wash well.

   b. Power up the fluorometer (there may be a delay before the light source ignites).

c. Allow 20-30 min for the fluorometer to warm up and stabilize. During the warm up period, the recorder pen should slowly move up scale and then stabilize.

d. After the baseline has stabilized, perform the fluorometer adjustment (see Section V.H.3). The recorder should be on during this procedure. The recorder must be switched to "Reverse polarity".

e. Set the function switch to "No damping". The other fluorometer settings will now depend on the sensitivity desired. Set the reagent baseline to "0" chart units or slightly above after all reagents have reached the fluorometer flow cell.

f. Fluorometer settings and dialyzer sizes for measuring three concentration ranges of CN^- are presented in the following table.

EXPERIMENTAL CONDITIONS FOR MEASURING THREE CONCENTRATIONS RANGES OF CN^-

Sensitivity (Range)	Dialyzer (Inches)	CN^- (μM/L)	Apertures (Sample)	Apertures (Reference)	Standard Calibrations (Settings)
I	6	$\leq$4,000	#2	#4	~7.0
II	12	$\leq$ 200	#3	#4	~4.5
III	24	$\leq$ 10	#4	#4	~3.0

3. Fluorometer Adjustment

a. Set the Sample Aperture to Position C.

b. Set Standard Calibration control to 0.00.

c. Set Baseline control to 0.00.

d. Set Reference Aperture to Position 3.

e. Set Function switch to the Reference Position.

f. Adjust the light pipe to give a recorder reading of ~15 chart units. If 15 chart units cannot be reached, use the highest reading possible.

g. Reset Function switch to No Damping.

h. Reset Sample and Reference Apertures and Standard Calibration for desired sensitivity.

i. Adjust baseline to 0 chart units.

4. Blood samples should be collected in heparinized syringes or Vacutainers (B-D). Immediately transfer a 250 ± 3 µL volume of blood to a 0.5 mL autoanalyzer sample cup containing 5 ± 0.05 µL of 4-DMAP solution (0.5 g/dL). Cap, mix, and set aside (room temperature) to be assayed later for total CN^-. Immediately transfer 1 ± 0.1 mL of blood to a 1.5 mL centrifuge tube and centrifuge for 1 min at 15,000 rpm. Quickly separate plasma from erythrocytes, transfer to an autoanalyzer sample cup and assay for free CN^-. It is essential that this assay be performed as soon as possible to minimize the loss of recoverable CN^-.

   After all plasma samples have been analyzed, replace the first diluent with that specified for total CN^- and assay all blood samples that were previously set aside.

   Upon completion of analysis; all plasma, whole blood samples, and disposable transfer pipettes which have come in contact with the samples will be discarded as infectious waste using the methods described in FSSP SOP MREF-55. Turn off the Technicon® pump after rinsing the manifold for 10 min with water, then pump the manifold dry for 15 min. Release and remove the pump platen. Release tension on pump tubes by lifting the tube clamp bar. Discard the infectious waste container using the methods described in FSSP SOP MREF-55.

I. Data Calculations and Analysis

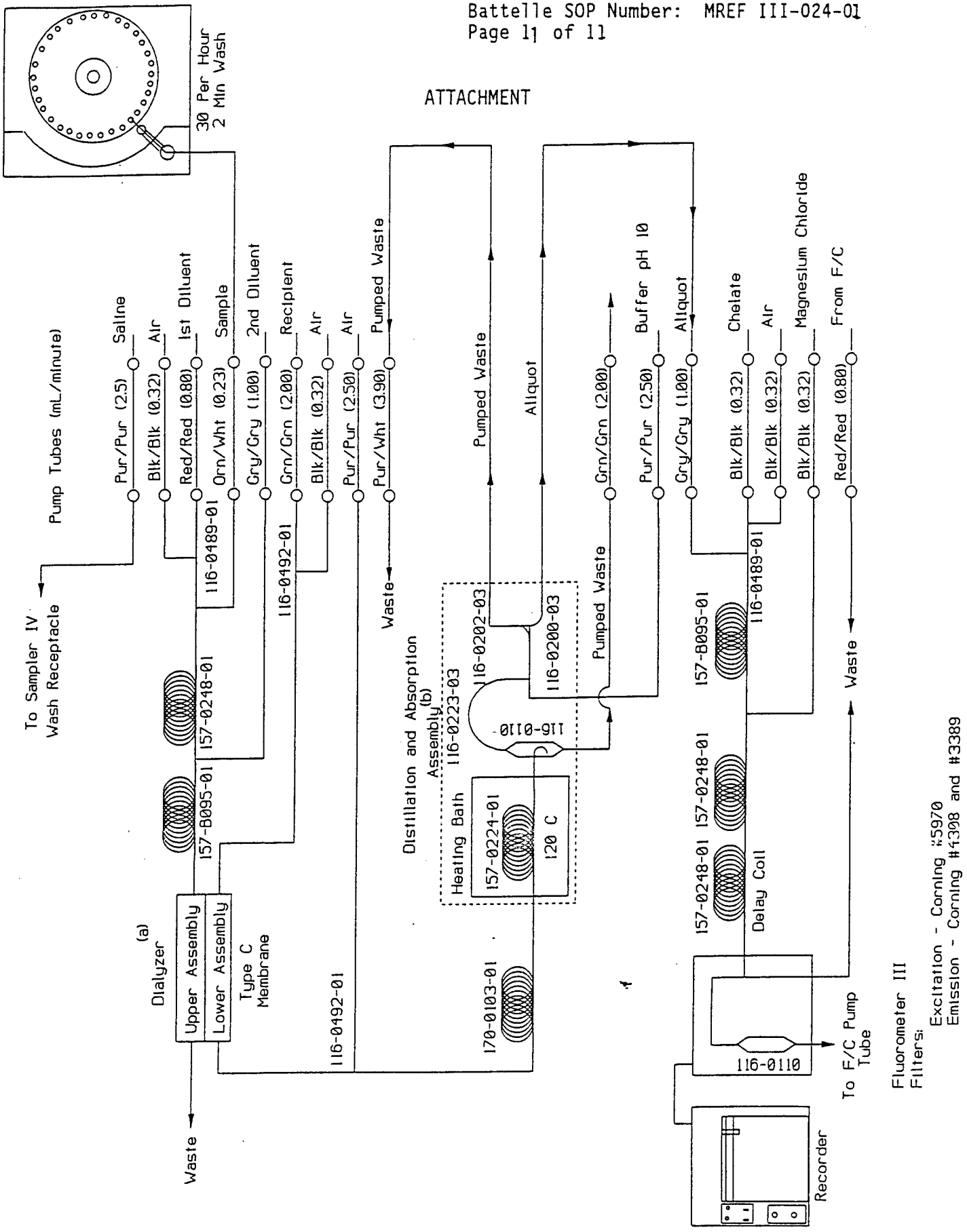
A linear curve is obtained for the CN^- standard range when mM/L values are plotted versus fluorescent peak height.

J. Quality Control

Each step in the analysis of standards and samples must be done in a reproducible manner to achieve good precision and accuracy. This includes sample preparation and instrument operation. A set of working standards, encompassing the desired range of CN^- concentrations, is assayed at the beginning and end of each working day. A control sample is assayed after each group of five unknowns to provide a measure of daily precision.

If the initial standard curve data are acceptable ($r \geq 0.98$) and the peak heights of CN^- control samples are within the established control ranges, accept the sample data. If a CN^- control sample is outside of the established control range, then reanalyze the CN^- control level in question. If after reanalysis, the duplicate CN^- control sample is still outside the established control range, then recalibrate the assay and reanalyze all samples that were assayed after the last acceptable CN^- control. If the standard curve data are unacceptable, the problem must be identified and the sample set reanalyzed. All sample sets must be bracketed by two control samples that are within the established control range to be acceptable.

ATTACHMENT


Sampler IV
30 Per Hour
2 Min Wash
Pump Tubes (mL/minute)
To Sampler IV
Wash Receptacle
Pur/Pur (2.5) Saline
Blk/Blk (0.32) Air
Red/Red (0.80) 1st Diluent
Orn/Wht (0.23) Sample
Gry/Gry (1.00) 2nd Diluent
Grn/Grn (2.00) Recipient
Blk/Blk (0.32) Air
Pur/Pur (2.50) Air
Pur/Wht (3.90) Pumped Waste
116-0489-01
116-0492-01
Waste
Distillation and Absorption Assembly (b)
Heating Bath 116-0223-03
157-0224-01
120 C
116-0202-03
116-0200-03
116-0110
Pumped Waste
Aliquot
Grn/Grn (2.00)
Pur/Pur (2.50) Buffer pH 10
Gry/Gry (1.00) Aliquot
Blk/Blk (0.32) Chelate
Blk/Blk (0.32) Air
Blk/Blk (0.32) Magnesium Chloride
Red/Red (0.80) From F/C
157-8095-01
157-0248-01
Delay Coil
Waste
116-0110
To F/C Pump Tube
Recorder
Fluorometer III
Filters:
Excitation - Corning #5970
Emission - Corning #4308 and #3389
(a) Dialyzer
Upper Assembly
Lower Assembly
Type C Membrane
157-8095-01
157-0248-01
116-0492-01
170-0103-01
Waste

ATTACHMENT C-III.

METHOD FOR ANALYSIS AND IDENTITY CONFIRMATION OF P-AMINOPROPIOPHENONE OR P-AMINOHEPTANOPHENONE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

METHOD FOR ANALYSIS AND IDENTITY CONFIRMATION OF p-AMINOPROPIOPHENONE OR p-AMINOHEPTANOPHENONE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

A. Statement of Work: This method describes the method for the quantitative analysis and identity confirmation of p-aminopropiophenone (PAPP) or p-aminoheptanophenone in an injectable solution. The prepared sample is analyzed by high performance liquid chromatography (HPLC). The sample preparation and analysis methods detailed here were developed in support of on-going tasks at the MREF.

B. Equipment: Freezer, labels, autosampler vials, first aid kit, plastic-backed, absorbent paper, brown paper, squirt bottles, wiping tissues, beakers, volumetric flasks, bottles, pipettes, pipette bulbs, tissue paper, 0.45 μm filter apparatus, laboratory coat, safety shoes, safety glasses, spatula, syringes, and latex gloves.

The HPLC analytical system will consist of the following: Waters 600 Controller, Waters 600E pump, Waters 715 autosampler, Waters 991 diode array ultraviolet/visible detector, analytical columns and guard columns as specified in Sections D.1.a and E.1.a and an electronic data system.

Equivalent equipment may be substituted for the above listed components.

C. Area Set Up: A laboratory area with a chemical fume hood, and an analytical balance in a vented enclosure will be used to prepare calibration standards and perform sample preparation and dilution procedures.

The hood areas for solvent handling are covered with plastic-backed, absorbent paper. All materials for sample preparation are located in or near the hood area.

D. Procedures

1. PAPP or PAHP Quantitation:

   a. Instrument Preparation: The HPLC is prepared for use with the following recommended initial settings. The optimum operating conditions shall be determined by the analyst:

Column - 25 cm x 4.6 mm inside diameter (I.D.) Supelco LC-18-DB Column with 5 μm partial size, or equivalent.

Guard Column - 2.0 cm x 4 mm I.D. Supelco LC-18-DB Guard Column with 5 μm partial size, or equivalent.

Mobile Phase: 45 percent buffer/55 percent acetonitrile.

Mobile Phase Flow Rate: 1.5 mL/min.

Injection Loop: 20 μL volume.

Detector Wavelength: 270 nm.

Absorbance Units Full Scale (A.U.F.S.): 2.0.

b. Column Conditioning: The column needs to be conditioned until a stable baseline is observed. This typically requires allowing mobile phase to flow through the column for approximately 30 min at the normal flow rate. This conditioning is required so the stationary phase can be equilibrated with the mobile phase, producing a homogeneous environment.

2. Reagent/Solution Preparation:

a. Mobile Phase Buffer: Accurately weigh 2.02 $\pm$ 0.01 g sodium heptane sulfonate onto weighing paper. Quantitatively transfer the powder into a 1-L volumetric flask containing approximately 500-mL of HPLC grade water. Add 1.00 $\pm$ 0.01 mL of concentrated acetic acid, and dilute to a volume of 1-L with HPLC grade water. Filter the resulting solution through a 0.45 μm filter.

b. Mobile Phase: The mobile phase is prepared by mixing the mobile phase buffer prepared in Section D.2.a with acetonitrile (organic) in the ratio specified in Section D.1.a. The ratio of buffer to organic in the mobile phase may require adjustment depending upon the analytical column used. This adjustment will be determined prior to conducting any analyses. The final ratio will be recorded in the laboratory record book for the task.

c. Benzophenone Internal Standard Stock Solution: The benzophenone stock solution is prepared from pure crystalline benzophenone. Accurately weigh 10 $\pm$ 0.1 mg of benzophenone into a 10-mL volumetric flask. Dilute to

volume with the mobile phase prepared in Section D.2.b. Store in the freezer at -20 C pending analysis.

d. PAPP Stock Solution: The PAPP stock solution is prepared from pure crystalline PAPP supplied by Chemsource Inc. Standards should be prepared in a range of concentrations which bracket the nominal concentrations of the diluted samples. An example of a suitable dilution scheme follows. Exact concentrations and volumes may vary depending upon protocol and equipment used.

1.0-mg/mL PAPP Stock Solution: Accurately weigh 10 ± 0.1 mg of PAPP into a 10-mL volumetric flask. Add approximately 5-mL of the vehicle which is used to prepare the dosing solution, such as PEG 200. Mix well using a vortex mixer and sonication. PEG 200 is a viscous liquid and requires a considerable amount of mixing to get PAPP to dissolve. Dilute to volume with PEG 200 and mix again.

e. Preparation of PAPP Analytical Standards:

(1) 0.100-mg/mL Analytical Standard: Aliquot 1.00 ± 0.02 mL of the PAPP stock solution prepared in Section D.2.d. into a 10 mL volumetric flask containing 1.0 ± 0.02 mL of the benzophenone internal standard prepared in Section D.2.c. and dilute to 10 mL with the mobile phase. Aliquot the standard into appropriately labeled autosampler vials. Store in the freezer at -20 C pending use.

(2) 0.070-mg/mL Analytical Standard: Aliquot 0.70 ± 0.014 mL of the PAPP stock solution Section D.2.d. into a 10 mL volumetric flask containing 1.0 ± 0.02 mL of the benzophenone internal standard prepared in Section D.2.c. and dilute to 10 mL with the mobile phase. Aliquot the standard into appropriately labeled autosampler vials. Store in the freezer at -20 C pending use.

(3) 0.050-mg/mL Analytical Standard: Aliquot 0.50 ± 0.01 mL of the PAPP stock solution Section D.2.d.into a 10 mL volumetric flask containing 1.0 ± 0.02 mL of the benzophenone internal standard prepared in Section D.2.c. and dilute to 10 mL with the mobile phase. Aliquot the standard into appropriately labeled autosampler vials. Store in the freezer at -20 C pending use.

(4) 0.020-mg/mL Analytical Standard: Aliquot 0.20 ± 0.004 mL of the PAPP stock solution Section D.2.d.into a 10 mL volumetric flask containing 1.0 ± 0.02 mL of the benzophenone internal standard prepared in Section D.2.c. and dilute to 10 mL with the mobile phase. Aliquot the standard into appropriately labeled autosampler vials. Store in the freezer at -20 C pending use.

(5) 0.000-mg/mL Analytical Standard: Aliquot 1.0 ± 0.02 mL of the benzophenone internal standard prepared in Section D.2.c. into a 10 mL volumetric flask and dilute to 10 mL with the mobile phase. Aliquot the standard into appropriately labeled autosampler vials. Store in the freezer at -20 C pending use.

f. PAHP Stock Solution: The PAHP stock solution is prepared from pure crystalline PAHP. Standards should be prepared in a range of concentrations which bracket the nominal concentrations of the diluted samples. An example of a suitable dilution scheme follows. Exact concentrations and volumes may vary depending upon protocol and equipment used.

120 mg/mL PAHP Stock Solution: Accurately weigh 30 ± 0.1 mg of PAHP into a reacti-vial and add 0.25 ± 0.02 mL of PEG 200. Mix well using a vortex mixer and sonication. PEG 200 is a viscous liquid and requires a considerable amount of mixing to get PAHP to dissolve.

1.20 mg/mL PAHP Stock Solution: Aliquot 0.100 ± 0.001 mL of the PAHP stock solution prepared in Section D.2.f. into a 10 mL volumetric flask containing approximately 6 mL of mobile phase prepared in Section D.2.b Mix well using a vortex mixer and then dilute to 10 mL with the mobile phase. Re-mix the solution before use. Store in the freezer at -20 C pending use.

g. Preparation of PAHP Analytical Standards:

(1) 0.480-mg/mL Analytical Standard: Aliquot 4.00 ± 0.02 mL of the PAHP stock solution prepared in Section E.5.b. into a 10 mL volumetric flask containing approximately 6 mL of mobile phase prepared in Section D.2.b Mix well using a vortex mixer and dilute to 10 mL with the mobile phase. Re-mix the solution and aliquot the standard into appropriately labeled autosampler vials. Store in the freezer at -20 C pending use.

(2) 0.360-mg/mL Analytical Standard: Aliquot 3.0 ± 0.02 mL of the PAHP stock solution Section D.2.f.into a 10 mL volumetric flask containing approximately 6 mL of mobile phase prepared in Section D.2.b Mix well using a vortex mixer and then dilute to 10 mL with the mobile phase. Re-mix the solution and dilute to 10 mL with the mobile phase. Aliquot the standard into appropriately labeled autosampler vials. Store in the freezer at -20 C pending use.

(3) 0.240-mg/mL Analytical Standard: Aliquot 2.0 ± 0.02 mL of the PAHP stock solution Section D.2.f.into a 10 mL volumetric flask containing approximately 6 mL of mobile phase prepared in Section D.2.b Mix well using a vortex mixer and then dilute to 10 mL with the mobile phase. Re-mix the solution and dilute to 10 mL with the mobile phase. Aliquot the standard into appropriately labeled autosampler vials. Store in the freezer at -20 C pending use.

(4) 0.120-mg/mL Analytical Standard: Aliquot 1.0 ± 0.02 mL of the PAHP stock solution Section D.2.f.into a 10 mL volumetric flask containing approximately 6 mL of mobile phase prepared in Section D.2.b Mix well using a vortex mixer and then dilute to 10 mL with the mobile phase. Re-mix the solution and dilute to 10 mL with the mobile phase. Aliquot the standard into appropriately labeled autosampler vials. Store in the freezer at -20 C pending use.

(5) 0.000-mg/mL Analytical Standard: Aliquot 10 mL of mobile phase prepared in D.2.b into an appropriately labeled autosampler vial. Store in the freezer at -20 C pending use.

h. Collection and Storage of Samples: Samples are collected in vials and stored in the freezer at -20 C pending analysis.

i. Sample Preparation: Before analysis, the samples are diluted to a concentration within the calibration range of the instrument. The same dilution procedures are used to dilute the samples as were used to prepare the calibration standards. An example of a suitable dilution scheme for a 1 mg/mL PAPP stock solution follows.

Aliquot 0.50 ± 0.01 mL of the stock solution into a 10 mL volumetric flask containing 1.0 ± 0.02 mL of the benzophenone internal standard and dilute to 10 mL with the mobile phase. Aliquot the standard into appropriately labeled autosampler vials. Store in the freezer at -20 C pending analysis.

3. Analysis:

a. Calibration: Instrument calibration is performed when quantitation of samples is required by injecting 20 μL of each analytical standard prepared in Sections D.2.c. and D.2.g. using an autosampler. A complete set of calibration standards will be analyzed prior to analysis of samples. Once the calibration of the instrument has been checked for linearity, the samples are analyzed with at least every sixth sample being a calibration standard which can be used to verify the stability of the instrument. A complete set of calibration standards is analyzed following the last sample. All calibration standards analyzed are used to develop a complete calibration curve for quantitation of the samples. Samples that yield responses less than the calibration range will be reported as less than the lower quantitation limit (lowest standard analyzed). Any sample response that exceeds the largest calibration standard will be reported as greater than the upper quantitation limit (highest calibration standard). Any sample analyzed higher than the calibration range must be diluted to within the calibration range and reanalyzed.

b. Analysis of Samples: Samples and calibration standards are analyzed using the same procedure. At least every sixth analysis should be a standard.

c. Calculations:

(1) The calibration data are calculated using an internal standard correction method. The corrected data is the analyzed using a linear regression model in the form of $y = ax + b$.

(2) Using a linear regression program, generate the slope(a), intercept(b), and correlation coefficient for the calibration data.

(3) Enter the peak area of the analyte divided by the peak area of the internal standard as the abscissa (y-value) and the corresponding standard concentration as the ordinate (x-value).

(4) Enter each data point obtained from the calibration standards and calculate percent relative standard deviation (% RSD) between replicate standards. Do not include the blank in the calibration calculations as this will weigh the regression toward zero.

(5) If a regression program is not available, use the following calculations:

$$b = \frac{[(\Sigma y)(\Sigma x^2)-(\Sigma x)(\Sigma xy)]}{[n(\Sigma x^2)-(\Sigma x)^2]}$$

$$a = \frac{[n(\Sigma xy)-(\Sigma x)(\Sigma y)]}{[n(\Sigma x^2)-(\Sigma x)^2]}$$

$$r = \frac{[n(\Sigma xy)-(\Sigma x)(\Sigma y)]}{[(n(\Sigma x^2)-(\Sigma x)^2)^{1/2}(n\Sigma(y^2)-(\Sigma y)^2)^{1/2}]}$$

where,

$y = ax + b$
a = slope
b = y-intercept
r = correlation coefficient
x = concentration of agent in mg/mL
y = peak area analyte
n = number of replicates

(6) Identify the analyte and internal standard peaks in the sample chromatograms; record the peak areas. Using the regression values calculated from the calibration data, calculate the measured concentration for each sample using the above formula.

4. Post Analysis

a. Column Clean-up: At the end of each analysis day, the column needs to be flushed to remove contaminants and buffers. Flush the column with 100 percent HPLC grade water for 30 min at a flow rate of 2 mL/min followed by a mixture of 33:33:43 acetonitrile, methanol, and water for approximately 15 min at a flow rate of 2-mL per min. Then flush the column with 100 percent acetonitrile for approximately 15 min at a flow rate of 2 mL/min.

b. Instrument Shut-Down: When the instrument is not to be used for extended periods of time, the system must be shut down following manufacturer's instructions to ensure column life and instrument stability. The column clean-up procedure is followed, and the column is stored with 100 percent acetonitrile wetting the stationary phase.

E. PAPP or PAHP Identification:

1. Instrument Preparation: The HPLC is prepared for use with the following recommended initial settings. The optimum operating conditions shall be determined by the analyst:

   a. Column - 25 cm x 4.6 mm inside diameter (I.D.) Supelco LC-18-DB Column with 5 μm partial size or equivalent.

      Guard Column - 2.0 cm x 4 mm I.D. Supelco LC-18-DB Guard Column with 5 μm partial size or equivalent.

      Mobile Phase: 60 percent buffer/40 percent acetonitrile.

      Mobile Phase Flow Rate: 1.0 mL/min.

      Injection Loop: 20 μL volume.

      Detector Wavelength: Diode Array Scan between 190 and 399 nm.

      Absorbance Units Full Scale (A.U.F.S.): 2.0.

   b. Column Conditioning: The column needs to be conditioned by allowing mobile phase to flow through the column for approximately 30 min. The conditioning is performed so that the stationary phase can be equilibrated with the mobile phase, producing a homogeneous environment.

2. Reagent/Solution Preparation:

   a. Mobile Phase Buffer: Accurately weigh 2.02 $\pm$ 0.01 g sodium heptane sulfonate onto weighing paper. Quantitatively transfer the powder into a 1-L volumetric flask containing approximately 500-mL of HPLC grade water. Add 1.00 $\pm$ 0.01 mL of concentrated acetic acid, and dilute to a volume of 1-L with HPLC grade water. Filter the resulting solution through a 0.45 μm filter.

   b. Mobile Phase: The mobile phase is prepared by mixing the mobile phase buffer prepared in Section E.2.a. with acetonitrile in the ratio specified in Section E.1.a.

   c. 1.0-mg/mL PAPP Stock Solution: Accurately weigh 10 $\pm$ 0.1 mg of PAPP into a 10-mL volumetric flask. Add approximately 5-mL of the mobile phase

prepared in E.2.b. Mix well using a vortex mixer. Dilute to volume with the mobile phase and mix again.

d. Preparation of PAPP Analytical Standards:

0.100-mg/mL Analytical Standard: Aliquot 1.00 ± 0.02 mL of the PAPP stock solution into a 10 mL volumetric flask and dilute to volume with the mobile phase prepared in Section E.2.b. Aliquot the standard into appropriately labeled autosampler vials. Store in the freezer at -20 C pending analysis.

e. Collection and Storage of Samples: Samples are collected in 2-mL GC vials and stored in the freezer at -20 C pending analysis.

f. Sample Preparation: The samples are diluted to a concentration within the calibration range of the instrument before analysis. The same dilution procedures are used to dilute the samples as were used to prepare the calibration standards. Aliquot 0.50 ± 0.01 mL of the PAPP stock solution into a 10 mL volumetric flask and dilute to volume with the mobile phase prepared in Section E.2.b. Aliquot the standard into appropriately labeled autosampler vials. Store in the freezer at -20 C pending analysis.

3. Analysis:

a. Calibration: Instrument calibration is performed when identification of samples is required by injecting 5 μL of the analytical standards prepared in Section E.2.d. The diode array spectrum of the PAHP reference standard peak is used to create a reference library designated as PAHP.

b. Analysis of Samples: A 5 μL aliquot of the PAPP dosing stock prepared in Section E.2.f. is injected, and the retention and spectral data of the resulting chromatograph are entered into the PAPP library for comparison.

4. Column Cleanup and Instrument Shutdown: See Section D.4.

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ATTACHMENT C-IV.

METHOD FOR THE DETERMINATION OF HYDROXYLAMINE (NH_2OH) IN AQUEOUS SOLUTIONS BY TITRATION

METHOD FOR THE DETERMINATION OF HYDROXYLAMINE (NH_2OH) IN AQUEOUS SOLUTIONS BY TITRATION

A. Statement of Work:

This method describes the titrimetric analysis of hydroxylamine by oxidation with potassium bromate ($KBrO_3$) in the presence of hydrochloric acid (HCl):

$$BrO_3^- + NH_2OH \xrightarrow[\text{cold}]{(HCl)} Br^- + NO_3^- + H^+ + H_2O$$

At the end of the reaction free bromine appears:

$$BrO_3^- + 5Br^- + 6H^+ = 3Br_2 + 3H_2O$$

In this equation, 1 mole of bromate equates to 3 moles of bromine. The excess bromine is then determined by the addition of potassium iodide (KI) followed by titration with sodium thiosulfate ($Na_2S_2O_3$):

$$2I^- + Br_2 = I_2 + 2Br^-$$

Quantitation of NH_2OH is accomplished by determining the difference in the amounts of excess bromine between a water blank and NH_2OH sample (see Section H). The titrimetric analysis described in this method was developed in support of pharmacokinetics studies conducted at Battelle's Medical Research and Evaluation Facility (MREF).

B. Materials to be Used:

Unless specified, the materials used should be of reagent grade or better quality. Vendors are not specified since multiple suppliers are available for all materials.

ASTM type II water, hydroxylamine hydrochloride ($NH_2OH \bullet HCl$, grade I), concentrated HCl, $KBrO_3$, KI, $Na_2S_2O_3$, starch (potato, arrowroot, or soluble), salicylic acid ($C_7H_6O_3$) and zinc chloride ($ZnCl_2$) or sodium propionate ($C_3H_5O_2Na$) and sodium azide (NaN_3), chloroform ($CHCl_3$), anhydrous potassium dichromate ($K_2Cr_2O_7$, primary standard quality), and concentrated sulfuric acid (H_2SO_4).

C. Equipment:

Fume hood, analytical balance, spatula, beakers (25 and 50-mL), class "A" volumetric pipettes (2, 5, and 20-mL), Pasteur pipettes, pipette bulbs, 125-mL or 250-mL erlenmeyer flasks with ground-glass stoppers, class "A" micro burets (5-mL capacity; ± 0.01 mL tolerance), buret stand, volumetric flasks (50, 500, 1000, and 2000-mL), beakers (50 and 500-mL), graduated cylinders (10, 50, and 100-mL), glass bottles, prescored glass ampules (50-mL capacity), flame sealing torch, magnetic stirrer, magnetic stirring bars, mortar and pestle, ice bucket, tissue paper, laboratory coat, safety glasses, and latex gloves.

D. Sampling and Storage:

Bromine is very volatile, and hence such operations should be conducted at low temperature in a fume hood using conical flasks fitted with ground-glass stoppers.

E. Reagent Preparation:

1. Potassium bromate solution, 0.1 M. Using a 2-L volumetric flask, transfer 33.40 g $KBrO_3$ to approximately 1 L of distilled water. Mix the solution to dissolve the $KBrO_3$ and then dilute to 2 L with distilled water. Mix the solution again to ensure homogeneity. The estimated shelf life of this solution is 1 year after the date of preparation. Store in a glass bottle at room temperature.

2. Standard sodium thiosulfate solution, 2 N. Using a 2-L volumetric flask, transfer 632.44 g $Na_2S_2O_3$ to approximately 1 L of freshly boiled distilled water. Mix the solution to dissolve the $Na_2S_2O_3$ and then add 2 mL (0.1 percent v/v) of $CHCl_3$. Freshly boiled distilled water and $CHCl_3$ are used to minimize bacterial decomposition. Dilute to 2 L with freshly boiled distilled water and mix again to ensure homogeneity. Store solution for at least 2 weeks. This initial storage is necessary to allow oxidation of any bisulfite ions present. The estimated shelf life of this solution is 1 year after the date of preparation. Store in a glass bottle at room temperature. Prior to use, the sodium thiosulfate solution (titrant) must be standardized against potassium dichromate as follows:

   Using a 500-mL volumtric flask, transfer 4.902 g of anhydrous $K_2Cr_2O_7$ to approximately 250 mL of distilled water. Mix the solution to dissolve the $K_2Cr_2O_7$ and then dilute to 500 mL with distilled water. Mix the solution again to ensure homogeneity. This preparation yields a 0.2 N $K_2Cr_2O_7$ solution. The estimated shelf life of this solution is 1 year after the date of preparation. Store at room temperature in a sealed container. To 80 mL of distilled water, add (with constant

stirring) 1 mL of concentrated H_2SO_4, 5.00 mL of 0.2 N $K_2Cr_2O_7$ (using a 5-mL volumetric pipet), and 1 g of KI. Allow reaction mixture to stand 6 min in the dark. Prepare a 0.2 N $Na_2S_2O_3$ solution by diluting 5 mL of 2 N $Na_2S_2O_3$ solution (using a 5-mL volumetric pipet) to 50 mL in a volumetric flask with distilled water. Vortex the solution to ensure adequate mixing. Titrate the $K_2Cr_2O_7$ reaction mixture with 0.2 N $Na_2S_2O_3$ until the yellow color of the liberated iodine is almost discharged. Add 5 drops of starch indicator solution and continue titrating until dark blue color disappears. Record the volume of titrant used to the nearest 0.01 mL.

$$\text{Normality of } Na_2S_2O_3 = \frac{\text{mL } Na_2S_2O_3 \text{ consumed}}{2.5}$$

3. Hydroxylamine hydrochloride control solution, 50 mg/mL. Using a 2-L volumetric flask, transfer 100.00 g of $NH_2OH \bullet HCl$ to approximately 1 L of distilled water. Mix the solution to dissolve the $NH_2OH \bullet HCl$ and the dilute to 2 L with distilled water. Mix the solution again to ensure homogeneity. Glass ampules (50-mL capacity) are filled with 20 mL aliquots of the $NH_2OH \bullet HCl$ control solution after which the ampules are flame-sealed. The estimated shelf life of this solution is 1 year after the date of preparation. Store at room temperature.

4. Starch indicator solution. Dissolve 5 g of starch in enough cold water to form a thin paste in a mortar and grind thoroughly with a pestle to form a uniform mixture. Pour mixture into 1 L of boiling distilled water and stir until thoroughly mixed. Let solution settle overnight. Decant clean supernate into a glass bottle. Preserve with 1.25 g of $C_7H_6O_3$ and 4 g of $ZnCl_2$, or 4 g of $C_3H_5O_2Na$ and 2 g NaN_3 per L of starch solution. The estimated shelf life of this solution is 1 year after the date of preparation. Store at room temperature.

5. Potassium iodide solution, 10 percent (w/v). Transfer 50 g KI into 500 mL of distilled water. Mix solution to dissolve the KI. The estimated shelf life of this solution is 1 year after the date of preparation. Store in a glass bottle at room temperature.

6. Hydrochloric acid, 5 M. Using a 250-mL graduated cylinder, slowly add 250 mL of concentrated HCl to 250 mL of distilled water. The estimated shelf life of this reagent is 1 year after the date of preparation. Store in glass bottle at room temperature.

F. Procedure:

1. Determination of NH_2OH in aqueous solutions:

   a. Using a 2-mL volumetric pipet, transfer 2 mL of NH_2OH solution to a 125-mL erlenmeyer flask with a stir bar and a ground-glass stopper. Using a 20-mL volumetric pipet, transfer 20 mL of 0.1 M $KBrO_3$ to the flask containing the NH_2OH solution. Swirl gently to mix and place in an ice bath. Position the ice bath on a magnetic stirring plate and begin stirring the solution.

   b. Using a 50-mL graduated cylinder, transfer 40 mL of 5 M HCl to the flask containing the reaction mixture. Stopper the flask and allow the reaction mixture to set for 15 min with constant stirring to equilibrate the temperature.

   c. After the reaction mixture has set for 15 min, add 10 ml of KI solution using a pipet. Immediately stopper the flask and stir for one min. Remove the reaction flask from ice bath and place on a magnetic stirring plate and stir while titrating with standardized 2 N $Na_2S_2O_3$. Titrate the reaction mixture until the yellow iodine color almost disappears, then add 5 drops (using a Pasteur pipet) of starch indicator and continue titrating until the disappearance of blue color is observed and a clear, colorless solution remains. Placing a white piece of paper underneath the reaction flask will assist in determining the endpoint. Record the volume of titrant used to the nearest 0.01 mL.

   d. Determine the blank by repeating the process as described above in the titration of NH_2OH solution. The titration endpoint of a blank occurs when the blue color disappears and an opaque, white solution remains.

G. Analysis of Samples:

Three separate aliquots of samples, a control, and a blank should be titrated for each analysis.

H. Calculations:

The NH_2OH concentration is calculated as follows:

$$\text{mg } NH_2OH \text{ per mL} = \frac{(A - B) \times N \times 69.49}{3 \times P \times (\text{mL of sample})}$$

where,

A = mL titrant for blank
B = mL titrant for sample
N = normality of $Na_2S_2O_3$
P = purity of NH_2OH
69.49 = molecular weight of $NH_2OH \bullet HCl$

I. Quality Control:

Each step in the analysis of samples and blanks must be done in a reproducible manner to achieve good accuracy and precision to 2 decimal places. A control sample is analyzed with each unknown to provide a measure of daily precision.

If the NH_2OH control value is within the established confidence limits, accept the sample data. If the NH_2OH control value is outside of the established confidence limits, then reanalyze a new aliquot of the NH_2OH control solution. If after reanalysis, the NH_2OH control value is still outside of the established confidence limits, then the problem must be identified and the sample set reanalyzed.

J. References:

Vogel, A.I., A Textbook to Quantitative Chemical Analysis, 5th ed., Longman Scientific & Technical, U.K., 1989, ch. 10.135.

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ATTACHMENT C-V.

A Shelf-life Stability Study on 8-[(4-Amino-1-methylbutyl)amino]-5-(1-hexyloxy)-6-methoxy-4-methylquinoline DL-Tartrate, WR-242511AE, BM05816

Report No. 738
Addendum 1 to Report No. 720
7 August 1991

Contract No. DAMD17-91-C-1135
SRI International Project No. 2653

For
Headquarters, U.S. Army Medical
Research and Development Command
Office of the Surgeon General
Washington, D.C. 20314

Preface

This report was prepared at SRI International, 333 Ravenswood Avenue, Menlo Park, California 94025, under U.S. Department of the Army Contract No. DAMD17-91-C-1135, SRI International Project No. 2653, "Analytical, Characterization, and Stability Studies of Chemicals, Bulk Drugs, and Drug Formulations". This project is supported by the Division of Experimental Therapeutics, Walter Reed Army Institute of Research (WRAIR), Walter Reed Army Medical Center, U.S. Army Medical Research and Development Command. Dr. Robert R. Engle of the Department of Medicinal Chemistry is the project monitor.

This work was conducted in the Life Sciences Division by chemists John Pick, Lee L. Do, and Robert Petesch II between 22 July and 2 August 1991.

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A Shelf-life Stability Study on 8-[(4-Amino-1-methylbutyl)amino]-5-(1-hexyloxy)-6-methoxy-4-methylquinoline DL-Tartrate, WR-242511AE, BM05816

Draft
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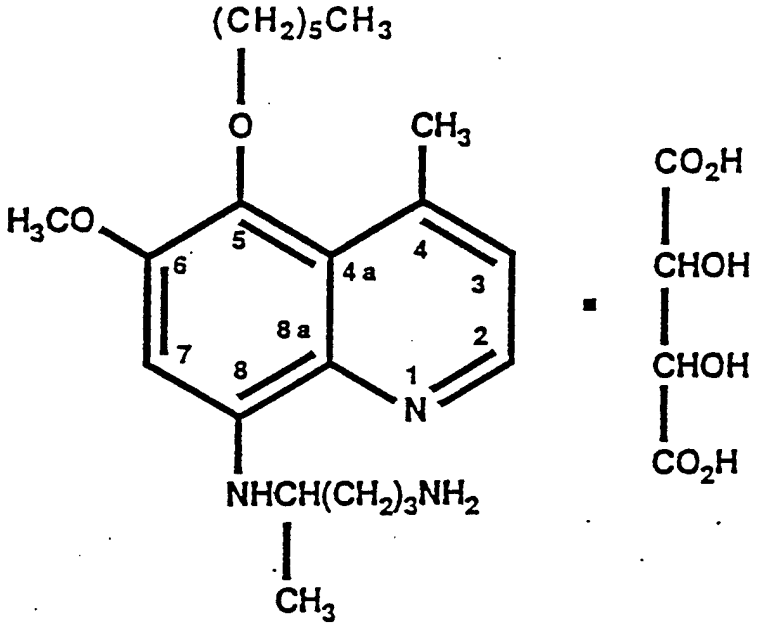

$C_{26}H_{41}N_3O_8$ M.W. = 523.64

Objective

The objective of this investigation is to determine the shelf-life stability of the subject sample when stored at room temperature (20-26°C) as outlined below in the experimental section.

Summary

This determination is the first in a series of periodic assays performed to ascertain the shelf life of the subject sample.

The subject material has been stored in a capped, amber-glass bottle at room temperature (20-26°C) under laboratory illumination.[1] Portions of the sample were removed after 3 and 6 months storage and stored in a freezer at -18°C until analyzed by thin-layer and high performance liquid chromatography.

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[1] Laboratory illumination means light from a standard fluorescent fixture (three 40-watt tubes) placed about 5 feet above the sample bottle. The bottle is illuminated for about 12 hrs/day on weekdays and not at all on weekends. Over a 7-day period, the bottle is illuminated for about 60 hrs. The standard fluorescent tube emits a continuous spectrum that ranges from 350 to 750 nm and exhibits maximum outputs near 440 and 540 nm.

Stability information was based on comparisons of data from the subject samplings to those from the reference. Integrity of the reference standard was based on spectral data.

Purities found for the 3- and 6-month samplings, 99.1±1.0% and 99.5±1.0%, respectively, are comparable to the purity of the reference, 99.2±1.0%, suggesting that no change has occurred in the subject samplings. TLC and HPLC profiles of the 3- and 6- month samplings showed no decomposition products, thus corroborating the initial purity results.

Experimental

The subject sample was received on 1 August 1990 and was stored at -18°C when it was not being analyzed. Experimental data are recorded in Notebook No. 10022.

I. Storage Conditions

A portion of the subject sample was transferred to an amber-glass, screw-capped bottle and stored at room temperature (20-26°C) under laboratory illumination[1] for this continuing shelf-life stability study. An initial sampling was taken at three months and stored at -18°C until a subsequent sampling was taken at six months, and both were then assayed.

II. Verification of Reference Standard by UV

The spectra (Figure 1 is representative) were recorded on a Cary I uv-vis spectrophotometer from 5 x 10^{-5}M solutions in 0.1N aqueous HCl. The molar absorptivities (ε_{max}, current) reported are averaged from four determinations (i.e. two iterations for each of two samplings) and are shown alongside the molar absorptivities reported in the original study (ε_{max}, orig.). Results indicate no measurable decomposition of the reference WR-242511AE.

λ_{max}	ε_{max}, original	ε_{max}, current
205nm	24900, s = 240	24400, s = 305
239	23300, s = 175	23300, s = 153
269	16500, s = 140	16600, s = 74
335	2370, s = 60	2340, s = 12

III. Thin-Layer Chromatography

Adsorbent: SiO_2-GF plates (Analtech, Inc., 5 x 20 x 0.025 cm)

Samples Spotted: WR-242511AE (t = 3 and 6 months); 300 µg (10 mg/300 µl MeOH)

Reference Spotted: WR-242511AE (t = 0); 300 µg (10 mg/300 µl MeOH)

Detection:

(a) UV, 254 nm

(b) Iodine vapor

Solvent Systems: (Solvent fronts traveled 12 cm ; all solvents are reagent grade)

(a) n-BuOH:HOAc:H_2O (15:3:2, v:v:v)

(b) MeOH:NH_4OH (10:1, v:v)

(c) MeOH:NH_4OH:$CHCl_3$ (22:3:75, v:v:v)

Results:

The samples and the reference are chromatographically identical. In solvent system (a), a major, yellow spot appeared at R_f 0.47 and minor spots at R_f 0.05 and 0.15 (this spot was detected with iodine vapor). In solvent system (b), a major, yellow spot appeared at R_f 0.33, and minor spots at R_f 0.02 and 0.10. In solvent system (c), a major, yellow spot was detected at R_f 0.67, with minor spots at R_f 0.00, 0.09, 0.24, 0.28, 0.39, and 0.93. All spots were visualized by uv except where noted. Every applied spot exhibited slight streaking when visualized with either detection method.

IV. HPLC

A. LC System

Column: DyChrom CN, 7 µ, 4.6 x 250 mm

Mobile Phase: 20% methanol:50% acetonitrile:30% 0.01M aqueous ammonium formate (pH adjusted to 3.0 with 88% aq. formic acid)

Flow Rate: 1.0 ml/min

Solvent Delivery System: Perkin-Elmer (PE) Series 4 LC

Injector: PE ISS-100 Autosampler; 10-µl injections from a 20 µl loop

Detection: PE LC-85B with LC-75 Autocontrol; UV, 220 nm

Integrator/Computer: PE SI-316/7500 with CHROM 3, Rev. 2.00

B. Procedure

Sample solutions containing ~3 mg WR-242511AE (t = 0, 3, and 6 months) per 10.0 ml methanol and an internal standard solution containing ~15 mg WR-171669AS in 50 ml methanol were prepared. Each sample vial for WR-242511 quantitation contained 500 µl sample solution and 500 µl internal standard solution; 10 µl aliquots were injected and chromatographed. Qualitative chromatograms were obtained from samples dissolved in the mobile phase. To show that the LC system was stability-indicating, a thermally decomposed reference was dissolved in methanol and chromatographed.

C. Calculations

Peak data from the references were used in the following equation to calculate area and height response factors (RF values).

$$RF = (A_r C_{is} V_{is}) \div (A_{is} C_r V_r P_r) \quad \text{Eq. 1}$$

where A_r = reference peak response

A_{is} = internal standard peak response

C_r = reference standard concentration

C_{is} = internal standard concentration

V_r = volume of reference standard assayed (500 µl)

V_{is} = volume of internal standard assayed (500 µl)

and P_r = reference standard purity (99.2%)

Subject sample peak data were used in the following equation to calculate the percent of WR-242511 decomposition, %D, in the stressed samplings.

$$\%D = 100(P_r - P_s) \div P_r \quad \text{Eq. 2}$$

where P_r is defined as described previously and P_s is the subject sample purity where

$$P_s = 100(A_s C_{is} V_{is}) \div (A_{is} C_s V_s RF) \quad \text{Eq. 3}$$

A_{is}, C_{is}, and V_{is} are the same as previously defined, and

A_s = subject sample peak response

C_s = subject sample concentration

V_s = volume of subject sample assayed (500 µl)

and RF = average area or height response factor

D. Results

Quantitative results (Table 1) show that the samplings and the reference are identical within experimental errors. The 3- and 6-month samplings contain 99.1, s = 0.8% and 99.5, s = 0.8% WR-242511, respectively. Chromatograms of the reference and the two samplings are overlaid in Figure 2.

Qualitative chromatograms of a blank, the reference (two samples), and the two samplings (two samples each), all overlaid in Figure 3, indicate that the system can detect impurities. Most chromatograms showed minor-to-trace components near 2.4, 3.4, 3.6, 3.8, 4.4, and 7.8 minutes. The 2.4-, 3.4- and 7.4- minute components

varied in amount even between samplings removed from the same container, suggesting sample inhomogeneity. However, aliquots from the same solution showed invariance, indicating that these variations are not caused by solution instability.

A chromatogram of a thermally decomposed sample (Figure 4) indicates that this LC system can also separate decomposed components. Peaks seen only in this thermally decomposed sample eluted at 2.7, 2.9, 4.7, 5.0, and 6.6 minutes.

Conclusion

Results from samplings that were stored 3 months and 6 months showed no evidence of decomposition. On this basis, the subject sample can be judged to be stable for at least 6 months under these storage conditions.

Table 1

WR-242511AE Shelf-life Stability Results
3 and 6 month samplings

Reference ID	Wt (mg)/ 10.0 ml	Ref. Area	Ref. Height	IS Area	IS Height	Area RF	Height RF
1	3.039	323913	25.4953	396691	17.3557	0.8171	1.4700
2	3.004	329488	25.8641	398452	17.4139	0.8371	1.5036
3	3.036	309291	24.3333	373784	16.4295	0.8289	1.4836
4	2.995	312048	24.5751	381441	16.7738	0.8307	1.4877
5	3.051	318415	24.6102	379534	16.4984	0.8362	1.4868
					Average =	0.8300	1.4863
					s =	0.0080	0.0120
					Relative s =	1.0%	0.8%

Wt (mg) of IS/50.0 ml
15.084

Reference Purity
99.2%

3 month Sample ID	Wt (mg)/ 10.0ml	Sample Area	Sample Height	IS Area	IS Height	Area Purity	Height Purity	Area % Decomp.	Height % Decomp.
1	3.037	324721	25.6439	391120	17.2025	99.4	99.6	-0.2	-0.4
2	2.990	316944	24.9915	383345	16.9831	100.5	99.9	-1.3	-0.7
3	2.966	310031	24.0296	384473	16.7225	98.8	98.3	0.4	0.9
4	3.042	311126	24.3031	378176	16.6062	98.3	97.6	0.9	1.6
5	3.034	322971	25.7148	390499	17.3131	99.1	99.4	0.1	-0.2
					Average =	99.2	99.0	0.0	0.2
					s =	0.8	0.9	0.8	1.0
					Relative s =	0.8%	1.0%		

Overall Average Purity = 99.1
s = 0.8
Relative s = 0.9%

Overall Average % Decomposition = 0.1
s = 0.9

6 month Sample ID	Wt (mg)/ 10.0ml	Sample Area	Sample Height	IS Area	IS Height	Area Purity	Height Purity	Area % Decomp.	Height % Decomp.
1	2.936	286955	23.0382	356356	16.0125	99.7	99.5	-0.5	-0.3
2	3.084	337764	26.5540	402156	17.6645	99.0	98.9	0.2	0.3
3	3.124	327946	25.9665	383414	17.0240	99.5	99.1	-0.3	0.1
4	3.002	333040	26.4894	399719	17.7477	100.9	100.9	-1.7	-1.7
5	2.901	288398	23.1100	367035	16.3507	98.6	98.9	0.6	0.3
					Average =	99.5	99.5	-0.3	-0.3
					s =	0.9	0.8	0.9	0.9
					Relative s =	0.9%	0.8%		

Overall Average Purity = 99.5
s = 0.8
Relative s = 0.8%

Overall Average % Decomposition = -0.3
s = 0.8

Data name	CURRENT#
Date/Time Stored	09:56 22-Jul-91
Abscissa Mode	NM
Photometric Mode	ABS
SBW	2.0
SAT	0.067
Data Interval	0.1111
Scan Rate	100.0000
Baseline Correct	ON
Status No. 1	
Status No. 2	

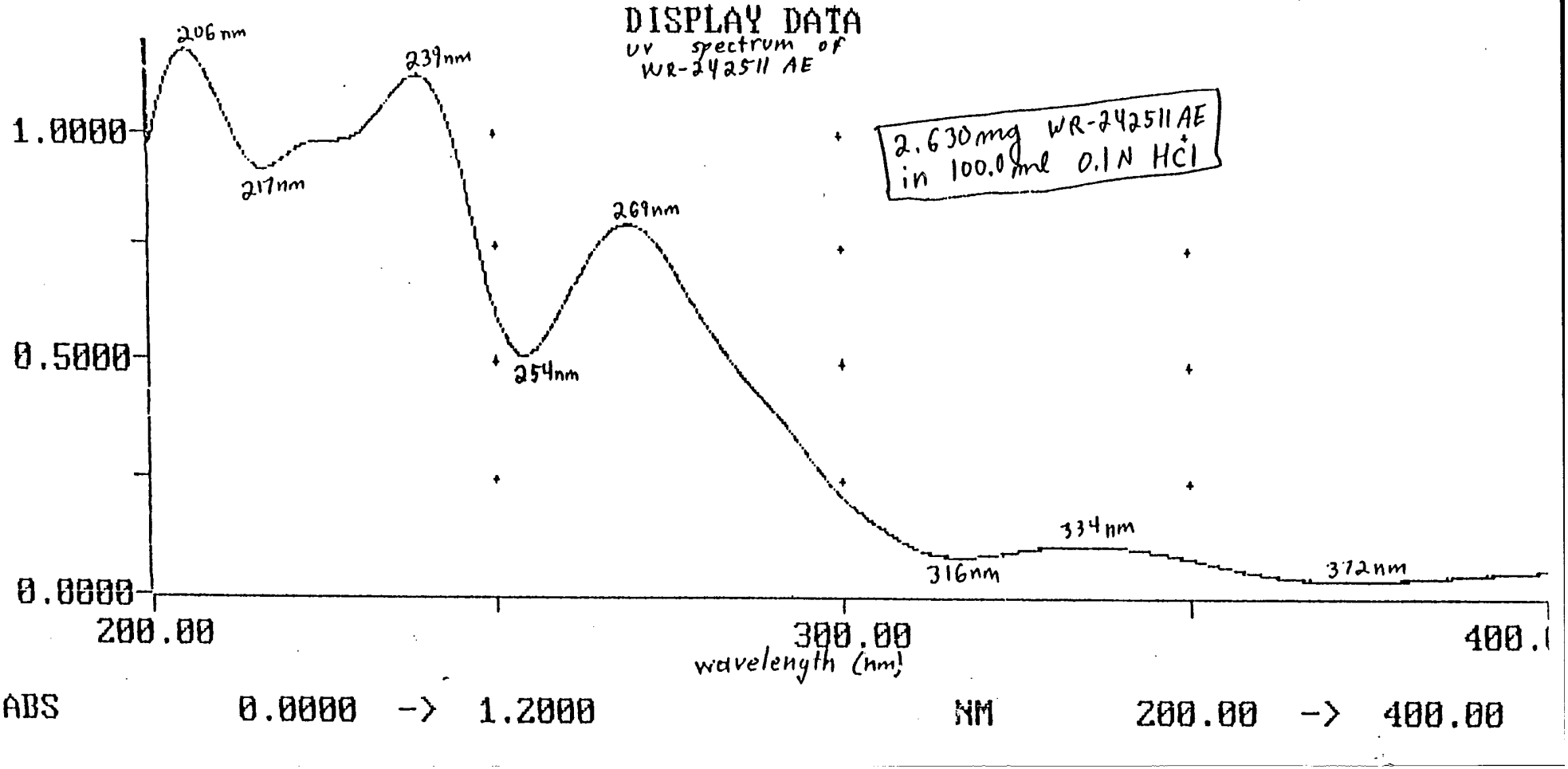
c. 0.263 mg/mL

0.3 mg/mL

c. 877 mL — 1 mL

1/11.4

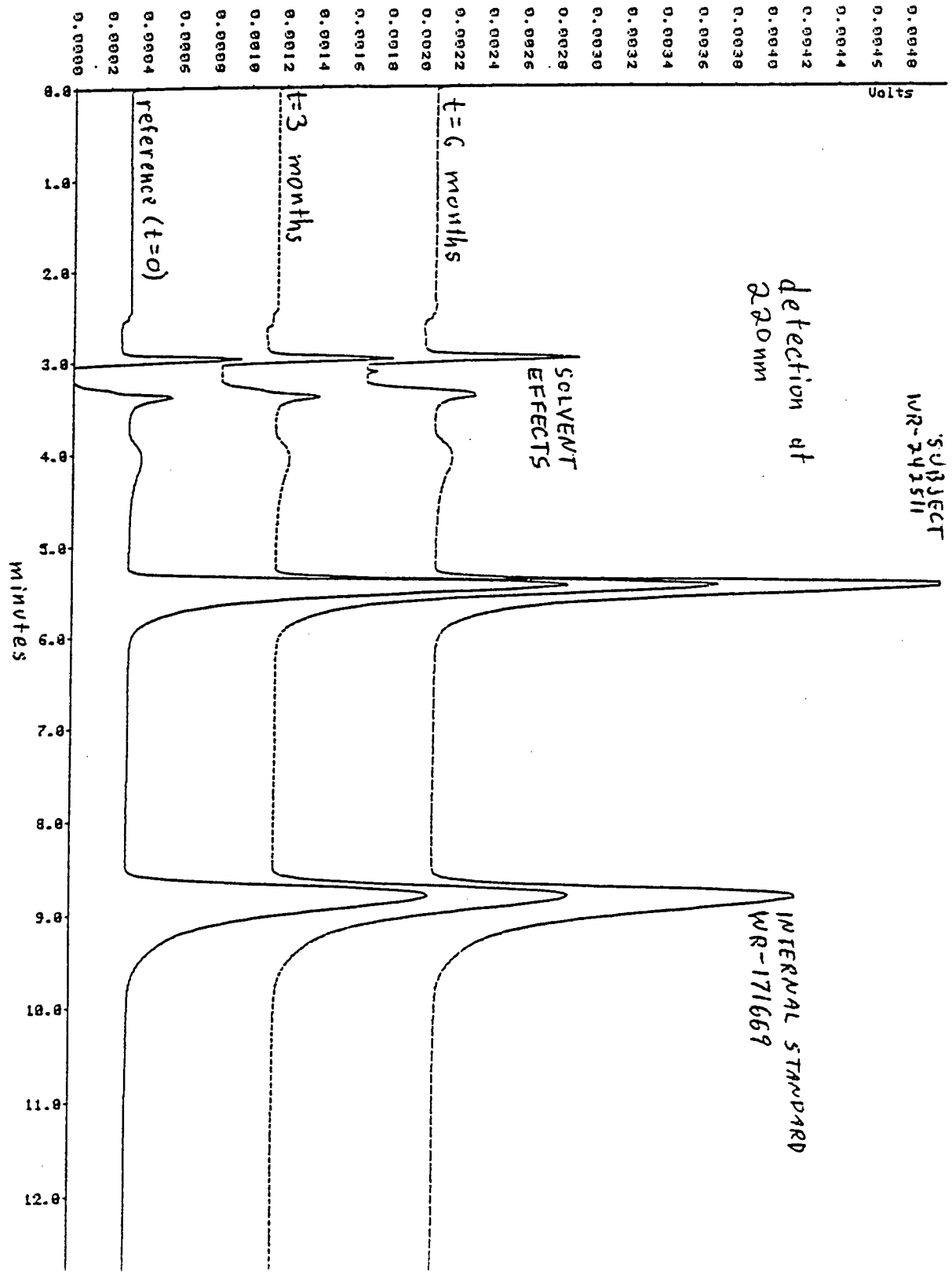
C-V.9



Data file: Q3S01V056S.01a John Pick Chromatograph # 2 Channel: CHAN:A
Collected on Thu Jul 25 12:05:22 1991 with LC Method # 6, WR242511AE
Description: WR242511AE-Ref1. Collection Peak Width: 5.5 - 13
Queue: 3, WR242511AE Vial #: 56 Inj #: 1
Last Processed on Fri Jul 26 10:01:43 1991 with LC Method # 7, WR-242511AEint

Data file: Q3S01V061S.01a John Pick Chromatograph # 2 Channel: CHAN:A
Collected on Thu Jul 25 13:15:31 1991 with LC Method # 6, WR242511AE
Description: WR242511AE-3mol. Collection Peak Width: 5.5 - 13
Queue: 3, WR242511AE Vial #: 61 Inj #: 1
Last Processed on Fri Jul 26 10:03:45 1991 with LC Method # 7, WR-242511AEint

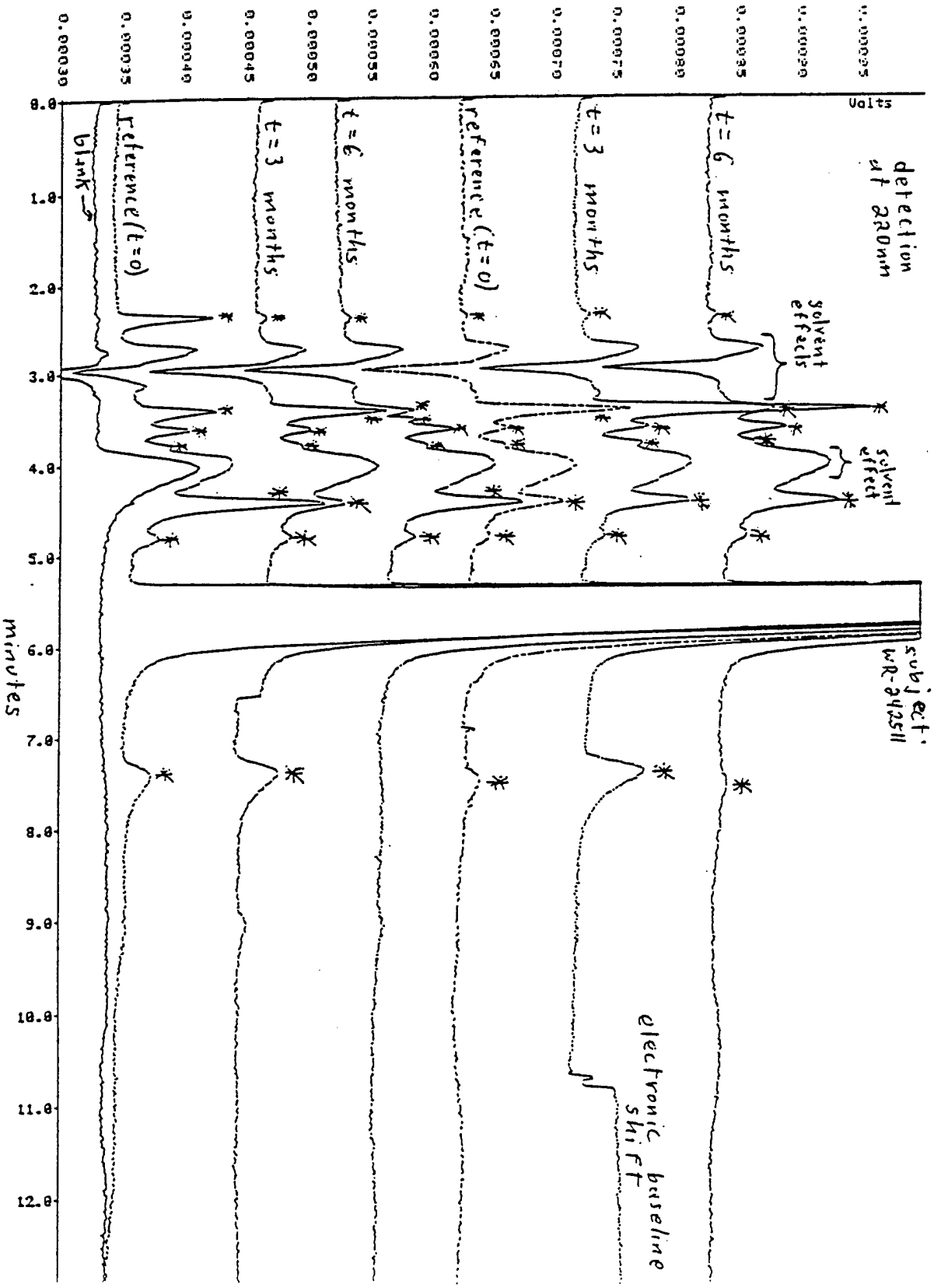
Data file: Q3S01V066S.01a John Pick Chromatograph # 2 Channel: CHAN:A
Collected on Thu Jul 25 14:25:32 1991 with LC Method # 6, WR242511AE
Description: WR242511AE-6mol. Collection Peak Width: 5.5 - 13
Queue: 3, WR242511AE Vial #: 66 Inj #: 1
Last Processed on Fri Jul 26 10:05:46 1991 with LC Method # 7, WR-242511AEint

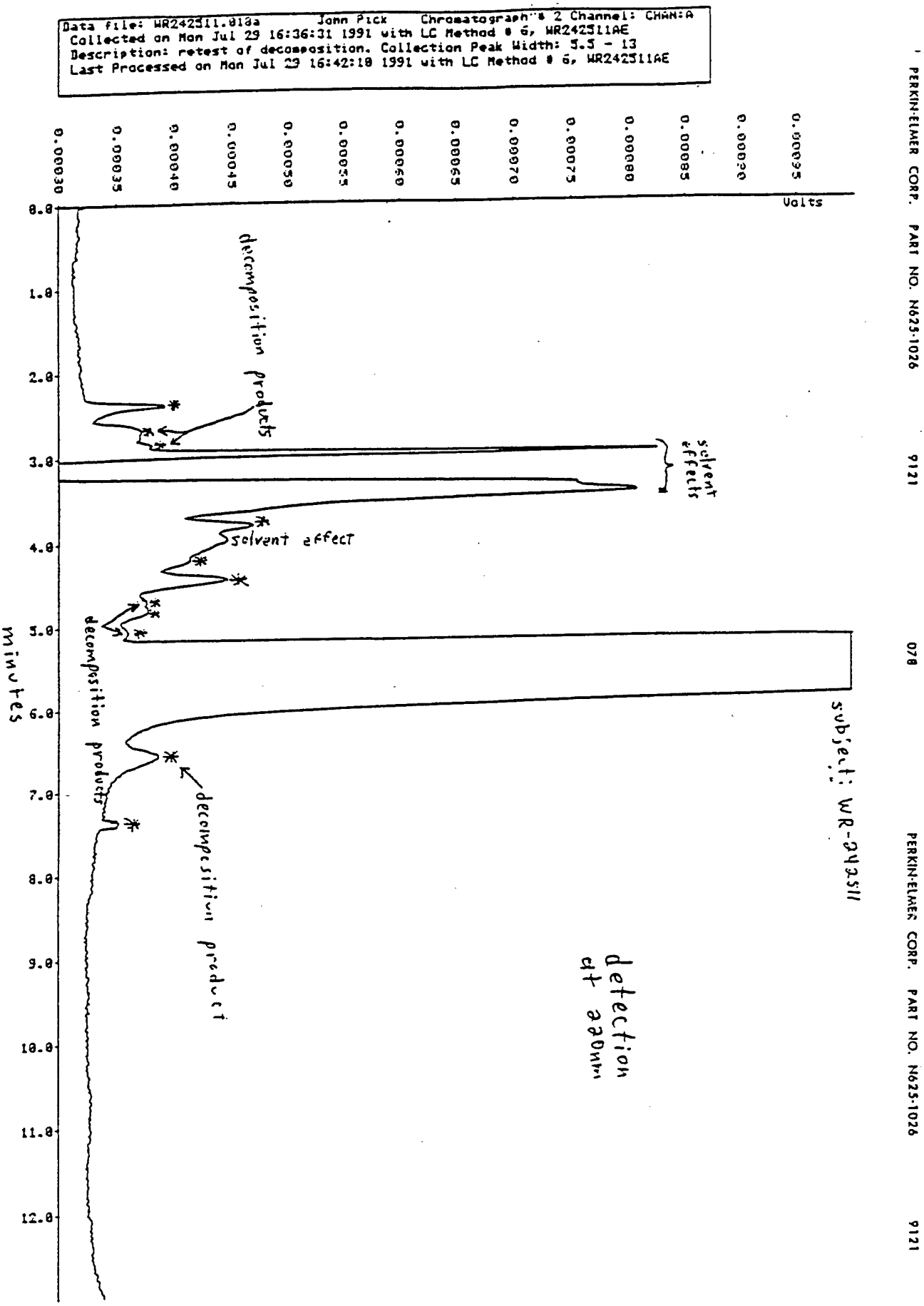


Data file: WR242511.026a Jonn Pick Chromatograph # 2 Channel: CHAN:A
Collected on Fri Aug 02 14:14:12 1991 with LC Method # 6, WR242511AE
Description: reference qualitative. Collection Peak Width: 5.5 - 13
Last Processed on Fri Aug 02 14:15:29 1991 with LC Method # 6, WR242511AE

Data file: WR242511.025a Jonn Pick Chromatograph # 2 Channel: CHAN:A
Collected on Fri Aug 02 13:45:19 1991 with LC Method # 6, WR242511AE
Description: 3-month qualitative. Collection Peak Width: 5.5 - 13
Last Processed on Fri Aug 02 13:46:28 1991 with LC Method # 6, WR242511AE

Data file: WR242511.027a Jonn Pick Chromatograph # 2 Channel: CHAN:A
Collected on Fri Aug 02 14:29:15 1991 with LC Method # 6, WR242511AE
Description: 6-month qualitative. Collection Peak Width: 5.5 - 13
Last Processed on Fri Aug 02 14:30:34 1991 with LC Method # 6, WR242511AE





* = impurity

Figure 4